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Microalgae for Wastewater Treatment and Biomass Production from Bioprospecting to Biotechnology

Sweiss, Mais

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Microalgae for Wastewater Treatment and Biomass Production from Bioprospecting to Biotechnology

Mais Ahed Sweiss

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Dedication

I would like to dedicate this thesis to my beloved mother, who is a brave, dedicated women with endless love for her family. To my father's soul who always encouraged me, filled me with hope, and taught me to follow my ambitions. To my brother and my sisters who supported me and showed me the joy in life.

Abstract

Improving wastewater (WW) treatment process is a major issue in different parts of the world. For a developed country like the UK where eutrophication is a problem that causes environmental and economical losses, and for a developing country like Jordan that is considered one of the most water scarce countries in the world, it is crucially important to improve the quality of the WW for safe reuse. Applying microalgae for WW treatment and biomass production is an economical and environmentally friendly method. However, this method has some challenges that need to be addressed, such as microalgae species selection, harvesting of the microalgae and the large area footprint.

In this research, the overall aim was to bioprospect for microalgae that are adapted to the wastewater treatment plants (WWTPs) and evaluate the obtained microalgae depending on specific criteria for a successful application in high rate algal ponds (HRAPs), then there were attempts to improve the phosphorus removal in microalgae to increase the efficiency of the treatment process and reduce the area footprint.

Bioprospecting for indigenous microalgae to the WW took place from January to May 2014. Water samples were collected from wastewater treatment plants (WWTPs) in the UK and Jordan. Eight different microalgae isolates were identified from each country. The results showed the *Chlorella*, *Scenedesmus* and *Desmodesmus* are common genera between the two countries and dominated the obtained isolates from the UK and Jordan. The isolates were identified using 18S rDNA and ITS1-5.8S-ITS2 DNA barcoding markers. It was difficult to identify some of the isolates at the species level, as the 18S rDNA is too conserved to differentiate between the closely related species and due to the relatively poor representation of algae in GenBank.

Then the obtained microalgae isolates were evaluated by their growth, efficiency in removing nutrients (i.e. nitrogen and phosphorus) and the settleability of the microalgae by gravity. Depending on the results the microalgae species were ranked to come up with some promising candidates to be applied on large scale. From the UK, Avonmouth_12 (Av_12) and Avonmouth_10 (Av_10) and from Jordan, Jordan_18 (Jo_18) and Jordan_29 (Jo_29) were distinguished in their performance in the WW.

Since phosphorus is a major cause of eutrophication in the fresh water and it is important to reduce the level of phosphorus in the released WW to the legally permitted limits, this

research aimed to study the possibility of improving phosphorus removal by microalgae. Using *Chlamydomonas reinhardtii* as a model to optimise the protocol to be applied in parallel with Av_12, which is a promising microalga isolate that has been applied on large scale in HRAPs in Beckington WWTP, the strategy was to overexpress a Phosphorus Starvation Response (*PSR1*) gene. The transformation process was successful in *C. reinhardtii* but not in Av_12. There was an enhancement of the specific phosphate removal rate in the transformed microalgae isolate CC-1010_B2 and CC-1010_A6 in comparison to the wild type strain CC-1010.

Abbreviations and acronyms

ANOVA	Analysis of variance
aph	Aminoglycoside 3`-phosphotransferase gene
atpB	ATPase beta-subunit
BBM	Bold Basal Medium
bp	Base pair
BPA	Bisphenol A
ca.	Circa (approximately, around)
CBCs	Compensatory base changes
CO_2	Carbon dioxide
cox1	Cytochrome oxidase 1
***cox2-3* spacer**	Cytochrome oxidase 2-cytochrome oxidase 3 intergenic spacer
cox3	Cytochrome oxidase 3
DHA	Docosahexaenoic acid
DIH_2O	Deionized water
DMSO	Dimethyl sulfoxide
DW	Dry weight
EBPR	Enhanced biological phosphorus removal
EDTA	Ethylenediamine-tetraacetic acid
EPA	Eicosapentaenoic acid
EPS	Extracellular polymeric substances
ETS	External transcribed spacer
FTIR	Fourier Transform Infrared
GOI	Gene of interest
h	Hour
HRAP	High Rate Algal Ponds
HSP70A	Heat shock protein 70A
HTL	Hydrothermal liquefaction
i1	Intron 1
IGR	Intergenic spacers
Inhab	Inhabitant

ITS	Internal transcribed spacer
kb	Kilo base pair
kt	Kilo ton
LSU	Large ribosomal subunit
MCM	Million cubic meters
MIC	Minimum inhibitory concentration
min	Minute
MPa	Megapascal
mt	Mutant
Nucleotide BLAST	Nucleotide Basic Local Alignment Search Tool
OD	Optical density
P	Phosphorus
PE	Population equivalent
PG	phosphatidylglycerol
Pi	Inorganic phosphorus
psaA	Code for Photosystem I P700 chlorophyll a apoprotein A1
psbA	Code for Photosystem II protein D1
PSR1	Phosphorus Starvation Response 1
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
rbcL	Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit
RBCS2	RuBisCO small subunit 2
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal RNA
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SQD1	UDP- sulfoquinovose synthase *SQD1*
SQD3	UDP- sulfoquinovose synthase *SQD3*
SQDG	Sulfolipids [sulfoquinovosyldiacylglycerol
SSU	Small ribosomal subunit
SVI	Sludge volume index
T_a	Annealing temperature

TA	TAP medium without phosphate
TAE	Running buffer (Tris Acetate EDTA)
TAGs	Triacyl glycerides
TAP	Tris acetate phosphate
TEP	Transparent extracellular particulate
TF	Transcription factor
T_g	Generation time
T_m	Melting temperature
TPBR	Tubular photobioreactor
TR	Transcription regulator
TRWR	Total renewable water resources
tufA	Encoding protein synthesis elongation factor Tu
UDP	Uridyl group
UV	Ultra violet
WSP	Waste Stabilization Pond
wt	Weight
WT	Wild type
WW	Wastewater
WWTP	Wastewater treatment plant
×g	Times gravity
yr	Year
μ	Specific growth rate
3′UTR	3′ untranslated region
NIR	Near-Infrared

Microalgae strains nomenclature

Av_2	Avonmouth_2
Av_3	Avonmouth_3
Av_7	Avonmouth_7
Av_10	Avonmouth_10
Av_12	Avonmouth_12
C. reinhardtii	*Chlamydomonas reinhardtii*
C. sorokiniana	*Chlorella sorokiniana*
CC-1010	Wild type *Chlamydomonas reinhardtii*, CC-1010 wild type mt+ [UTEX 90]
CC-1010_A-2	*Chlamydomonas reinhardtii strain* CC-1010 *PSR1* putative over expression line A-2
CC-1010_A-6	*Chlamydomonas reinhardtii strain* CC-1010 *PSR1* putative over expression line A-6
CC-1010_A-9	*Chlamydomonas reinhardtii strain* CC-1010 *PSR1* putative over expression line A-9
CC-1010_B-2	*Chlamydomonas reinhardtii* CC-1010 *PSR1* putative over expression line B2
CC-1010_B-18	*Chlamydomonas reinhardtii* CC-1010 *PSR1* putative over expression line B-18
CC-1010_B-34	*Chlamydomonas reinhardtii* CC-1010 *PSR1* putative over expression line B-34
CC-1010_Vector	*Chlamydomonas reinhardtii* CC-1010_WT transformed with empty pOpt-mVenus-Paro vector
CC-125	Wild type *Chlamydomonas reinhardtii*, CC-125 wild type mt+ [137c], parent of CC_4267
CC-4267 psr1-1	*psr1* mutant *Chlamydomonas reinhardtii*, CC-4267 psr1-1 mt-
Jo_2	Jordan_2
Jo_4	Jordan_4
Jo_12	Jordan_12
Jo_18	Jordan_18

Jo_23	Jordan_23
Jo_29	Jordan_29
Jo_34	Jordan_34
Jo_40	Jordan_40
S. obliquus	*Scenedesmus obliquus*
So_3	Somerton_3
So_15	Somerton_15
So_32	Somerton_32

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Chapter 1

General Introduction

1.1 Wastewater treatment

Water is an essential factor in a sustainable development, it underpins economic growth, environmental sustainability and poverty reduction. There are increasing demands on freshwater resources as a result of the population growth, industrialization, migration and urbanization. The demands on water are expected to increase by 55 % by 2050 (UNESCO, 2015) worldwide including in the UK, a developed country in Europe, and Jordan that is a developing water-scarce country in the Middle East. In the meantime, a vast amount of wastewater (WW) is produced every day around the world. For example, about 16 million cubic meters (MCM) of WW are treated daily by 9000 WW treatment plants (WWTPs) in the UK (Water UK, 2017), and around 0.375 MCM of treated WW are discharged per day from 33 WWTPs in Jordan (Spolaore et al. 2006; Ministry of Water and Irrigation-Jordan, 2016). It is extremely important to treat these large volumes of WW to prevent health and environmental problems such as eutrophication, as well as for safe reuse in agriculture and industry. This is especially relevant in regions that suffer from water scarcity such as Jordan where precipitation has declined by 20% in the last 80 years, and around 60% of fresh water resources are used for agriculture (Ministry of Water and Irrigation-Jordan, 2016).

According to (FAO, 2017a), WW is defined as "*Water which is of no further immediate value to the purpose for which it was used or in the pursuit of which it was produced because of its quality, quantity or time of occurrence. However, WW from one user can be a potential supply to another user elsewhere. Cooling water is not considered to be WW*"

Worldwide, WW is subjected to different treatment steps to remove different types of contaminants before being released into the environment. These treatments can range from a primary to a quaternary treatment step. When the WW first enters the WWTP, a preliminary screening step to remove large solids, grit and gravel takes place (Figure 1.1). This is followed by primary treatment, in which the contaminants that can be settled by gravity are removed. A secondary treatment is then applied in which the organic compounds are oxidized and biologically broken down by a mixed population of heterotrophic bacteria (De la Noüe, Laliberté and Proulx, 1992; Abdel-Raouf, Al-Homaidan and Ibraheem, 2012). After these treatments, the produced WW can be discharged to the environment. This WW is apparently clean water, but it has excess amounts of nitrogen and phosphate, and sometimes heavy metals (De la Noüe, Laliberté and Proulx, 1992). The overloads of nitrate, ammonium and phosphate in WW can be removed by tertiary treatment, while the removal of refractory organics and toxicants is achieved by a quaternary step (Oswald, 1988, pp. 305-328; De la Noüe, Laliberté and Proulx, 1992). Not all the WWTPs incorporate a tertiary treatment to remove the excess nutrients.

The excess output of nitrogen (N) and phosphorus (P) in the water causes eutrophication, resulting in the heavy growth of algae and plants. Eutrophication has negative consequences on the environment and society such as changing the dominant species; decreasing biodiversity; and affecting wild life, livestock watering, drinking water abstraction and treatment, water contact sports, tourism, angling and waterside property values (EA, 1998; EA, 2012a). In the UK, a surface area of 914 km^2 exhibits the symptoms of eutrophication (DEFRA, 2012). The eutrophication of freshwater in England and Wales costs the economy about £75–114 million per year, and an additional £54.8 million per year were spent to address the damage caused by eutrophication (Pretty et al. 2003). To avoid eutrophication and for safe reuse of WW it is important to treat the WW properly to remove nutrients such as nitrogen, phosphorus and other contaminants to improve the quality of the released WW.

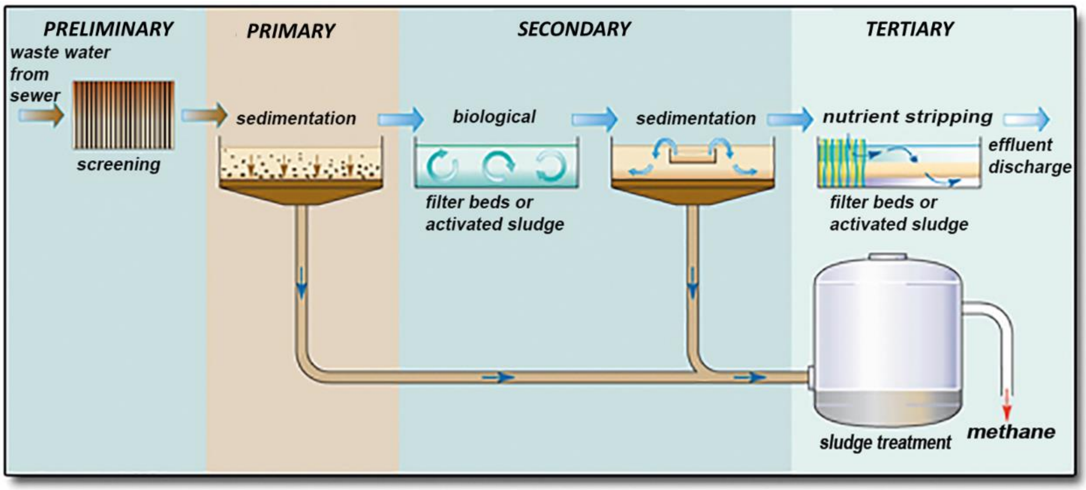

Figure 1.1: Diagram of the different WW treatment steps (replicated from The Open University web site http://www.open.edu/openlearn/science-maths-technology/science/environmental-science/energy-resources-water-quality/content-section-1.5.1)

1.1.1 Nitrogen and phosphorus removal from wastewater

As well as the negative impact of excess nutrients on the environment and the economy in general as mentioned above, water scarce countries such as Jordan suffer from this even more because there are limited water resources. Improving the quality of discharged WW for safe reuse in agriculture and industry is crucial. This can be accomplished by applying a tertiary treatment step for removing the extra nutrients such nitrogen and phosphorus. Diverse methods can be applied for removing nutrients from WW, each with its own advantages and disadvantages. The following section presents some of these methods, including one that we are particularly interested in the biological treatment of WW using microalgae.

Some conventional nitrogen removal methods

Nitrogen can be removed from WW by means of biological, physical and chemical methods (De la Noüe, Laliberté and Proulx, 1992). Conventionally, biological nitrogen removal in tertiary treatment is accomplished by bacteria and includes two steps: nitrification and denitrification within the aerobic zone. During nitrification, ammonia NH_3 is converted to nitrite (NO_2^-) by bacteria such as the members of genus *Nitrosomonas*. This nitrite is then converted to nitrate (NO_3^-) by autotrophic bacteria such as the genus *Nitrobacter* (U.S. EPA, 2002). In the second step, nitrate is reduced to gaseous nitrogen by heterotrophic bacteria such as *Flavobacterium* (Ni et al. 2017, pp368-418). This kind of biological treatment could have low efficiency if not enough carbon is present in the WW for denitrification (Blackburne, Yuan and Keller, 2008; Du et al. 2015).

One example of chemical nitrogen removal methods is ammonia stripping. This is a simpler and less expensive method than removing ammonia by nitrification and denitrification (U.S. EPA, 2000). In water, ammonia presents as ammonium hydroxide. The ammonia-stripping process includes the addition of lime to increase the pH to 10.8–11.5, allowing ammonium hydroxide ions to be converted into ammonia gas. This may affect air quality and cause pollution, and ammonia stripping will not remove nitrite or organic phosphates (U.S. EPA, 2000).

Some conventional phosphorus removal methods

Phosphorus can be removed from WW by chemical, physical and biological methods (Hoffmann, 1998). One of the physico-chemical methods for phosphorus removal is precipitation from the liquid by the addition of divalent or trivalent metal salts such as iron salts, aluminium salts, or lime (calcium oxide; Morse et al. 1998). Because chemical precipitation is relatively cheap and easy to operate and install in WWTPs, it is the most commonly used method for phosphorus removal in the UK (Ofwat, 2005). However it has some drawbacks, for instance the continuous consumption of aluminium or iron is considered less sustainable than biological methods (Hislop and Hill, 2011), these metals have been considered as pollutants themselves and included in the Water Framework Directive (WFD) (EA, 2012b). Chemical precipitation increases the amount of sludge produced (Ofwat, 2005) which increases the cost of handling, treatment and disposal of the sludge and using the iron salts for precipitation makes it harder to recover phosphorus from the sludge by using it as fertilizers (Levlin et al. 2002).

Biological phosphorus removal by bacteria (phosphorus accumulating organisms) was developed in the mid-1960s (Rybicki, 1997). It has been suggested that biological phosphorus removal is able to reduce the phosphate in discharged WW to around 0.1 mg/L (Barnard and Steichen, 2006). Like any technology, this method has some challenges that need to be overcome, such as that it cannot be applied to trickling filter systems (Morse et al. 1998). Another issue is that the bacteria can release the phosphorus if they are placed in anaerobic conditions again like the conditions in anaerobic digestion. In this case, the phosphorus will be released into the digestion liquors and returned back to the WWTP (Jardin and Pöpel, 1994).

1.1.2 Microalgae for nutrient removal

The term algae is used for "any organism that has chlorophyll a and a thallus that is not differentiated into roots, stem and leaves including the cyanobacteria that are prokaryotic organisms"(Lee, 1989, pp. 645 cited in Tomaselli, 2004, pp.3-19). Algae can be found as microalgae or as macroalgae (seaweeds). Macroalgae are multicellular algae and are classified depending on their pigments into three classes: *Chlorophycea*, macroalgae that have chlorophyll a and b that gives this group a green colour; *Phaeophyceae*, macroalgae with fucoxanthin and xanthophyll carotenoids that are responsible for a brown colour; and *Rhodophyceae*, red algae that have phycoerythrin and phycocyanin pigments

(Anbuchezhian, Karuppiah and Li, 2015, pp. 195-217). The term microalgae is used for microscopic algae and cyanobacteria (Tomaselli, 2004, pp.3-19). Microalgae are used for WW treatments.

The idea of applying microalgae for WW treatment in high rate algal ponds (HRAP) was first suggested by Oswald and Gotaas (1957). Oswald and his team at the University of California Berkeley revolutionised the mass algal culture technology by demonstrating that microalgae could be grown in large scale by integrating protein production and WW treatment. The principle of Oswald's team's technology was that domestic WW provides fresh water algae with nutrients and in turn algae provide oxygen for bacteria to break down organic matter in WW, and the bacteria provide the algae with carbon dioxide for growth. The produced algal-bacterial biomass represents a potential source of protein (Oswald and Gotaas, 1957; Goldman, 1979a).

Benefits of using microalgae in nutrient removal from WW

The first point to consider is that green microalgae can remove nutrients and toxic metals efficiently from a wide range of WW (De la Noüe, Laliberté and Proulx, 1992; Hoffmann, 1998; Chinnasamy et al. 2010; Cai, Park and Li, 2013; Kumar et al. 2015). There are extensive studies of algae growth in municipal WW (Woertz et al. 2009; Ji et al. 2013); agricultural WW such as dairy WW (Mulbry et al. 2008; Woertz et al. 2009) and piggery WW (Zhu et al. 2013); and industrial wastewater such as carpet mill WW (Chinnasamy et al. 2010), textile and dyeing WW (Sarwa, Vijayakumar and Verma, 2014.) and brewery effluent (Mata et al. 2012).

The second point is that applying green microalgae for WW treatment is considered ecologically safe, there is little or no need for adding chemicals and it does not lead to secondary pollution since the algal biomass would be harvested and the harvested biomass could be used for recycling nutrients (De la Noüe, Laliberté and Proulx, 1992; Hoffmann, 1998). Another environmental advantage of using microalgae for WW treatment is their photosynthetic CO_2 fixation, this means they can mitigate greenhouse gases by uptake CO_2 from the flue gas (Van Den Hende, Vervaeren and Boon, 2012; Razzak et al. 2013).

The third point to consider is the cost, in general, the implementation of tertiary treatment step will increase the cost of the WW treatment, for each additional step after the primary treatment step the cost will be doubled (Oswald, 1988, pp. 305-328 cited in De la Noüe, Laliberté and Proulx, 1992; Hoffmann, 1998). It has been argued that a complete tertiary treatment cost for the removal of both nitrogen and phosphorus will be about four times the cost of primary treatment. And inserting additional step for removing organic pollutant and or another step for removing the heavy metal this will increase the cost at least eight times higher than the primary treatment (Hoffmann, 1998; Oswald, 1988, pp.305-328; De la Noüe, Laliberté and Proulx, 1992). These available technologies consume significant amounts of chemicals and energy that make it costly process (Tchobanoglous and Burton, 1991, pp1334 cited in Hoffmann, 1998). From this point of view microalgae could be a less expensive option than some of the conventional treatment processes, since it would be able to consume the nitrogen and phosphorus while they are growing and they could be able to remove heavy metals at the same time (Rai, Gaur and Kumar, 1981; De la Noüe, Laliberté and Proulx, 1992; Kumar et al. 2015) and organic compounds (Redalje et al. 1989 cited in De la Noüe, Laliberté and Proulx, 1992; Ben Chekroun, Sánchez and Baghour, 2014) with little or no chemicals addition.

Drawbacks in applying microalgae to WW treatment

Microalgal based WW treatment technologies have some challenges that need to be addressed to overcome them, and to apply a successful microalgal WW treatment system. One of the major challenges of growing microalgae in large scales is the cost of harvesting, harvesting small suspended microalgae (less than 20 µm in size) is a costly process, and the biomass concentration is low (De la Noüe, Laliberté and Proulx, 1992; Olguí, 2003). Moreover, it is important to consider that microalgae are living microorganisms and so are affected by biotic factors such as predators, pathogens, and competition with other microalga and/or bacteria. Microalgal growth is also affected by abiotic factors for example pH, nutrient concentration, toxicants, temperature, and CO_2 and O_2 concentrations (De la Noüe, Laliberté and Proulx, 1992). Furthermore, microalgae need light optimisation (Goldman, 1979b), and mixing for their autotrophic growth (De la Noüe, Laliberté and Proulx, 1992). Thus, it is important to understand microalgae physiology to analyse and predict their response to different environmental factors (Benemann, 1989 cited in De la Noüe, Laliberté and Proulx, 1992) and also to be able to optimise growth and maximise productivity and efficiency in nutrient removal from WW (Olguı´, 2003).

To improve this technology and make it more cost effective, it is important to consider many factors when selecting microalgae for WW treatment such as: adaptation to the WW environment, tolerance of extreme temperatures, predation, grow fast and efficient in nutrients removal, easy to harvest to reduce the cost of harvesting, and if it has high added value product will make the process more economically viable. It is important to understand the physiology of the applied strain (Olguı´ 2003).

1.1.3 Systems for applying microalgae for WW treatment

Algae have been used in WW treatment around the world for around 60 years long time. It is important to improve this environmentally-friendly technology to achieve the best results that can be obtained. This could be done by studying the purpose (such as nutrients or heavy metal removal and biomass production) of applying microalgae. The conditions of the WWTP where the microalgae are to be applied needs to be studied, and then the system needs to be designed in such a way that it would give high efficiency during WW treatment. To make this technology economically favourable, it also needs to be low in cost.

Waste stabilisation ponds

Waste or wastewater stabilisation ponds (WSPs) are a common WW treatment system applied in thousands of small to medium-sized communities around the world such as New Zealand and the USA. It is suitable for rural areas and industries where land costs are not too expensive (Craggs et al. 2003 and Powell, 2009). WSPs are cost-effective and appropriate WW treatment processes. They are simple and inexpensive in construction and operation, and are effective in terms of organic carbon and pathogen removal, but not in nutrient removal (Craggs et al. 2003 and Powell, 2009).

Here is a brief overview of WSPs (Figure 1.2 A): WSPs are large, man-made ponds in which WW is treated naturally, with the help of solar light, wind, microorganisms and algae. The ponds can be used individually or linked in a series for improved treatment. There are three types of ponds: anaerobic, facultative and aerobic (maturation), each with different treatment and design characteristics. Anaerobic ponds are 2–5 m in depth and are used in the primary treatment stage in which suspended solids are settled and degraded by anaerobic microorganisms (Tilley et al. 2014). Facultative ponds are 1-2.5 m deep unmixed ponds, they are part of the second treatment stage in WSPs in which further removal of the organic matter takes place (Tilley et al. 2014). In the top layer where oxygen is provided by natural diffusion, wind mixing and by algae to encourage aerobic bacteria to degrade organic compounds, and the released nutrients are assimilated by the algae (Oswald, 1988, pp.305-328; Tilley et al. 2014). The third type is the aerobic (maturation) pond, shallow (0.5-1.5 m) unmixed ponds with large surface area that facilitate light penetration. They are designed for pathogen removal, the effluent is very low in pathogens but rich in nutrients, but if algae were applied it can reduce the nutrients in the effluent (Tilley et al. 2014). WSPs do not achieve high nutrient removal consistently, and they have a low algal biomass productivity (Craggs et al. 2003).

High rate algal ponds

High rate algal ponds (HRAPs) were developed in California by Oswald and Gotaas in 1957 for urban WW treatment as it was mention in section 1.1.2. HRAP technology for treating WW has been applied in several WWTPs in north California such as St. Helena WWTP in 1967, in 1997 it was applied in Delhi (Craggs, Lundquist and Benemann, 2013, pp.153-163), and at the Christchurch WWTP in New Zealand (Craggs, Sutherland and Campbell, 2012) (Figure 1.2 B). HRAPs are shallow open channel raceway ponds, they range in depth between 0.2–0.8 m depending on the clarity of the WW and the climate. The ponds are mixed by paddlewheel at a horizontal velocity of 0.15–0.30 $m \cdot s^{-1}$ (Craggs, Lundquist and Benemann, 2013, pp.153-163).

HRAPs have been developed for efficient and low cost WW treatment technology, this technology achieves high performance in removing organic matter and nutrients such as nitrogen and phosphorus (García et al. 2006). With the addition of CO_2 it has been used to treat different types of WW such as agricultural and industrial WW (Oswald 1988, pp.305-328; Fallowfield, Martin and Cromar, 1999; Craggs, 2005, pp.282-310). In addition to urban WW treatment, HRAPs are very suitable for WW treatment in small rural communities because of their simplicity of operation in comparison to conventional technologies such as activated sludge facilities (García et al. 2006). An important challenge in applying HRAPs for WW treatment is the relatively large land area requirement in comparison with activated sludge. Furthermore, the algal performance is affected by the climate conditions such as temperature and light duration and intensity. For this reason many studies have been done on HRAP to improve them for the seasonal variations of climate (Picot et al. 1991; Picot et al. 1993; Sutherland et al. 2014a) and since it is an open pond system it has a risk of contamination and predation by zooplankton (Nurdogan and Oswald, 1995; Montemezzani et al. 2017).

Bioreactors

To try to reduce the area footprint for tertiary treatment, increase the efficiency in removing nutrients, enhance productivity and to control microalgal species by reducing contamination and grazing problems in WW treatment, some research has been carried out to develop closed systems for growing algae for WW treatment and biomass production (Molinuevo-salces, García-gonzález and González-fernández, 2010, Min et al. 2011; Arbib et al. 2013). Most closed systems were developed to maximise the surface/volume ratio and enhance productivity and efficiency of nutrient removal. A study was performed to compare the performance in nitrogen and phosphorus removal and biomass production from urban WWTP effluent by *Scenedesmus obliquus* (SAG 276-10) grown in a tubular photobioreactor (TPBR; Figure 1.3 C) and HRAP. The microalgal growth, productivity and nutrient removal for TPBR were higher than HRAP, however, the TPBR has a main disadvantage that it results in severe biofouling (Arbib et al. 2013). There are some disadvatages for applying TPBR when comparing it with HRAP such as the high investment cost, overheating could cause it to be 10–15 °C higher inside the TPBR, biofouling accumulation that reduces light penetration and accumulation of O_2 that could inhibit photosynthesis (Mata, Martins and Caetano, 2010; Arbib et al. 2013). An evaluation of the performance of TPBR and HRAP needs to consider some factors that could cause a variation such as the geographical location and alage species (Arbib et al. 2013).

Figure 1.2: Systems for applying algae WW treatments, **(A)** WSPs Mathura II WWTP in Mathura in India, taken by Google Earth in 2009 adapted from Saph Pani (2013). **(B)** One of the four WW treatment HRAP (1.25 ha) at Christchurch, New Zealand replicated from (Craggs, Lundquist and Benemann, 2013, pp.153-163). **(C)** Airlift TPBR with solar loop receiver working volume of 330 L for secondary effluent WW treatment in (WWTP) (Arbib et al. 2013)

1.1.4 Applying HRAP for WW treatment

HRAP technology is commonly applied in large-scale microalgae production around the world not only for WW treatment. It is also used in a wide range of algal farms to produce *Spirulina* in Chili (Ayala, Vargas and Cardenas, 1988, pp.229-236), California USA (Earthrise, 2017) and Spain (Jimenez et al. 2003); for CO_2 fixation trials in Japan (Matsumoto et al. 1995) and for both biofuel production and WW treatment (Craggs, Sutherland and Campbell, 2012).

Figure 1.3: Raceway ponds driven by paddle wheels at the Earthrise Spirulina, California, USA. The production plant has 37 *Spirulina* ponds, each covers 5000 m^2, and is mixed by 15.24 m paddlewheels in each pond (Earthrise, 2017). The photo is adapted from (Borowitzka and Moheimani, 2013, pp. 133-152).

As mentioned above, in comparison with bioreactors they are constructed from less expensive materials so they have a lower cost of construction (Jorquera et al. 2010) and require lower energy input (Rodolfi et al. 2009; Brennan and Owende, 2013, pp. 553-599), they are also easy to operate (Jorquera et al. 2010). However, they have many challenges that need to be overcome to make them more efficient in WW treatment and microalgae production and more economically applicable. Biotic factors include risk of contamination by bacteria, fungus, other microalgae, and predation risk by grazers (Mata, Martins and Caetano, 2010; Arbib et al. 2013). Whilst, Abiotic factors include: low light efficiency utilisation, lack of temperature control, poor gas distribution, seasonal variation of climate (Picot et al. 1991; Picot et al. 1993; Jorquera et al. 2010; Arbib et al. 2013; Sutherland et al. 2014a).

Biotic factors

Contamination is an essential issue that needs to be overcome when applying microalgae to WW for many reasons starting with the fact that WW has a community of microorganisms that could have bacteria, protozoa, pathogenic viruses and helminths (FAO, 2017b). In addition, microalgae are grown in HRAPs, open to the environment, air, wind, insects and other algae, and they are grown long-term for treating WW and supplied with CO_2, which could be another reason for contamination (Borowitzka and Moheimani, 2013, pp.133-152). Microalgae cultures could be contaminated with bacteria, viruses, zooplankton and other competitive microalgae species.

Phytoplankton-lytic bacteria can affect microalgal growth either by direct attack relying on cell-to-cell contact (Imai et al. 1995; Shunyu et al. 2006) or by indirect attack using extracellular compounds (Imai et al. 1995; Kang et al. 2005). Viral infection can significantly reduce algal cell density within a few days and cause changes in cell structure, diversity and succession. Viruses can infect both eukaryotic algae and prokaryotic cyanobacteria (cyano-phage) (Wang et al. 2013). Other microalgae contaminations can affect the growth and dominance of the species of interest by different mechanisms like direct cell contact, competition of resources and the allelopathy effect (Wang et al. 2013).

Open ponds rich in nutrients at neutral pH are very susceptible to zooplankton growth: ciliate (Rosetta and Mcmanus, 2003), cladocera, copepod (Frederiksen et al. 2006), and rotifer (Lurling and Beekman, 2006) are the common predatory species in the mass cultivation of microalgae. Rotifers and cladocerans feed on microalgae and reduce the performance of HRAP for WW treatment and algal production (Montemezzani et al. 2017). Zooplankton control is essential for efficient WW nutrient removal, algal productivity, and HRAP stability (Wang et al. 2013; Montemezzani et al. 2017).

Contamination with biological pollutants (viruses, bacteria, fungi, protozoa, and other microalgae) could be manged by biological or chemical control, filtration, or modifying growth conditions. However, these methods still need more development to overcome some deficiencies. It is worth mentioning that strain selection is still the most practical choice, to select microalgae strains that are resistant and tolerant to biological pollutants is important to maintain and manage the growing culture (Wang et al. 2013).

Abiotic factors

There are many abiotic factors that affect microalgae growth, productivity and efficiency in removing the nutrients from WW such as light, temperature, pH, gas exchange, nutrient availability, etc. (Park, Craggs and Shilton, 2011b; Cai, Park and Li, 2013). The following section will summarise the details of some of these factors.

Light

Light intensity and shading of cells affect the microalgal growth in the HRAP (Park, Craggs and Shilton, 2011a). In nutrient sufficient conditions with increasing light intensity, the photosynthetic process increases until it reaches the maximum growth rate and at this point, the photosynthesis system reaches the light saturation point. If light intensity continued to increase above this it will lead to photoinhibition (photooxidation) at which point light receptors become damaged and productivity and photosynthetic rate decreases (Melis, 1999; Park, Craggs and Shilton, 2011a). Li et al. (2012) concluded that the optimal light intensity varies among different species, from their study of the effect of light intensity on the removal of nutrients from WW and biomass accumulation for biodiesel production for two algal species, the optimal light intensity was found to be 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for *Chlorella kessleri* and *Chlorella protothecoides* respectively, and both of the species were able to remove nutrients efficiently under different light conditions (Li et al. 2012).

Another point to consider that can limit microalgal growth in HRAP is the shading effect. When the cell density increases the shading effect of those cells also increases, so less light would be available to cells in deeper places. It has been reported that when the algal concentration was as high as 300 g of total suspended solids per cubic meter, most of the photosynthetic active radiation was absorbed in the top 15 cm of the HRAP. This light utilisation limitation on the productivity in HRAP could be managed by modifying the WW depth, biomass concentration and enhancing the mixing pattern. Paddlewheels provide a degree of vertical mixing and help to expose algae to the light (Grobbelaar, 2009; Park, Craggs and Shilton, 2011b; Sutherland et al. 2014b).

Temperature

Temperature has a key effect on algal productivity and also WW properties (Park, Craggs and Shilton, 2011a). Since the productivity of algae reaches maximum at the optimum temperature, if nutrients and light are not limited, above the maximum temperature photorespiration reduces the overall algal productivity (Sheehan et al. 1998; Pulz, 2001; Hodaifa, Martınez and Sanchez, 2010; Park, Craggs and Shilton, 2011b). It has been reported that *Scenedesmus obliquus* has the highest biomass productivity at 30 °C, and at lower temperatures (20 °C–25 °C) and higher temperatures (35 °C) the productivity decreased (Martinez, Jiminez and El Yousfi, 1999). In another study, it was found that in addition to the growth, the temperature significantly affected the nutrient uptake and biomass composition of *Scenedesmus obliquus,* which was grown in olive mill WW as a culture medium (Hodaifa, Martınez and Sanchez, 2010). The optimum temperature is measured under non-limiting light and nutrient conditions, and it varies among species. Laboratory experiments and ecological observations have led to a distinction between microalgae depending on their optimal growth temperature: psychrophiles have optimal temperatures below 20 °C, whilst mesophilic (20 and 35 °C) and thermophilic microalgae have optimum temperatures above 35 °C (Soeder et al. 1985). Under nutrient- and light-limiting conditions the optimum temperature is different (Park, Craggs and Shilton, 2011a). Another point is that the change in temperature can cause a change in the WW properties such as pH, gas solubility and ionic equilibrium, and the response of different algal species to these effects is different.

pH

The optimum pH range of many algae is 8.2–8.7, but there are some species that can tolerate a more acidic or basic environment (Barsanti and Gualtieri, 2006, p.213) such as *Spirulina*, which predominates in highly alkaline water (Metting, 1996). Many factors play a role in controlling the pH of the pond in addition to the composition of the WW (ionic constituents and alkalinity), such as the respiration of algae and bacteria, algal productivity and efficiency of CO_2 addition (Heubeck, Craggs and Shilton, 2007; Park, Craggs and Shilton, 2011a; Mehrabadi, Farid and Craggs, 2017).

In HRAP changing the pH of the pond disrupts microalgae growth and metabolism by affecting the bioavailability of CO_2 for photosynthesis and the availability and uptake of nutrients. A pH range of 8–9 is crucial for the concentration of free CO_2 in the medium (Azov 1982). During photosynthesis the algae consume CO_2 and produce O_2, raising the pH to over 11 (García et al. 2006; Park, Craggs and Shilton, 2011a). This enhances nutrient removal because it can cause ammonia volatilisation and phosphorus precipitation with unchelated ferric ion, calcium and magnesium, but at the same time, less nutrients are available for algae to consume (Cromar, Fallowfield and Martin, 1996; García et al. 2006). Furthermore, at high pH, the concentration of free ammonia resulting from the ammonium ion is increased, which inhibits photosynthesis in algae, resulting in growth reduction, especially in WW where ammonium is the major nitrogen constituent (Azov and Goldman, 1982). In HRAP for WW treatment a nitrite concentration of above 50 mg·L^{-1} at pH 6.0— possibly due to an incomplete nitrification process—inhibited marine algae cultures (De Pauw, Bruggeman and Persoone, 1978 cited in Cromar, Fallowfield and Martin, 1996).

Gas exchange

Appropriate gas exchange needs to be applied in HRAP to achieve higher efficiency in nutrient removal and biomass production. Gas exchange is important to transfer CO_2 to cells and remove excess O_2 (Christenson and Sims, 2011). Removal of excess O_2 above the saturation point is important to prevent photoinhibition of the algae and for closed reactors—especially oxygen removal—is a difficult challenge (Carvalho, Meireles and Malcata, 2006; Christenson and Sims, 2011)

Three major forms of dissolved inorganic carbon exist in equilibrium: carbon dioxide (CO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). Algae can only directly utilise two of the three: CO_2 and HCO_3^- (Knud-Hansen et al. 1998; Christenson and Sims, 2011). Because of the mass transfer limitation, open ponds could be carbon limited. The addition of CO_2 into outdoor algal cultures improved productivity and was recommended by Azov (1982). The addition of CO_2 has many benefits such as avoiding high pH and thus inhibiting free ammonia toxicity, and increasing the availability of nutrients such as ammonium and phosphate. The addition of CO_2 also increases the carbon to nitrogen ratio and overcomes carbon limitation in WW (Mehrabadi, Farid and Craggs, 2017), and enhanced the productivity of algae in HRAP (Heubeck Craggs and Shilton, 2007; Park and Craggs, 2011; Sutherland et al. 2015; Mehrabadi, Farid and Craggs, 2017). Both O_2 release and CO_2 transfer can be done with high efficiency by the use of gas-liquid contactor reactors like the rotating biological contactors (RBCs) common in secondary WW treatment (Zeevalkink et al. 1979; Christenson and Sims, 2011).

Economic challenges

HRAPs is more economically viable than some of the conventional nutrient removal systems (Nurdogan and Oswald, 1995; Hoffmann, 1998; Olguı´, 2003) as mentioned above. Since it is an environmentally friendly method it is important to improve the HRAP system to make it more favourable by increasing the efficiency and reducing the cost. There are many areas that could be improved in HRAPs for nutrient removal from

WW. HRAPs require a large land area for construction, which could therefore, make them more suitable for rural areas where more land is available at a lower cost, as it could be a challenge to apply this system in urban areas (Hoffmann, 1998).

Harvesting microalgae from large scale WW treatment systems is a major challenge that needs to be considered to achieve an economical WW treatment process (Hoffmann, 1998). Microalgae are hard to harvest because they are small in size, the unicellular eukaryotic microalgae size ranges from 3–30 μm (Grima et al. 2003; Christenson and Sims, 2011; Barros et al. 2015), cells have a negatively charged surface that helps them to form a stable suspension in the water (Tenney et al. 1969; Grima et al. 2003; Park, Craggs and Shilton, 2011b) and they could be motile, making them hard to harvest (Henderson et al. 2008). Furthermore, algae cells have similar densities to the water, which makes settling of the microalgae from WW difficult (Craggs et al. 2011). The US Department of Energy (DOE) in 2016 reported that cost-effective harvesting methods are one of the technical challenges and barriers for economical biofuel production from microalgal biomass. Mechanical and chemical methods for harvesting such as centrifugation, dissolved air floatation (DAF), filtration and chemical flocculation are considered to have high energy demand and are too costly (Benemann and Oswald, 1996; Manheim and Nelson, 2013).

A variety of methods used for harvesting microalgae are summarised in Table 1.1, in this research we are interested in the gravity sedimentation method for harvesting since it is a simple, inexpensive and environment friendly method. It is worth noting that there is no universal harvesting method that could be applied to efficiently harvest all microalgae species. An efficient method should be designed depending on the culture medium, end product properties and cell characteristics e.g. morphology, size, etc. (Barros et al. 2015). It would be useful to research how to reduce the time and the area needed for WW harvesting.

Table 1.1: Summary of different methods for microalgal harvesting advantages and disadvantages. Modified from (Barros et al. 2015)

Harvesting method/principle	Advantages	Disadvantages
Chemical coagulation/flocculation The adhesion of fine particles into larger aggregates that agglomerate to form larger flocs settle faster leaving a clear supernatant. Coagulation involves pH adjustment or electrolyte addition, flocculation is based on the addition of cationic polymers (Papazi et al. 2010).	○ Simple and fast ○ No energy requirements	○ Chemical flocculants may be expensive and toxic to microalgal biomass ○ Recycling of culture medium is limited
Auto and bioflocculation Occur naturally in microalgal cultures by: Elevating the pH through photosynthesis, microalgae remove CO_2 dissolved in the culture medium, and related to the secretion of extracellular polymeric substances (EPS) (Christenson and Sims, 2011) or by flocculants produced by bacteria.	○ Inexpensive ○ Allows culture medium recycling ○ Non-toxic to microalgal biomass	○ Changes in cellular composition ○ Possibility of microbiological contamination
Gravity sedimentation Simple method in which microalgae settle by gravity, microalgal density is an important factor.	○ Simple and the inexpensive	○ Time consuming ○ Possibility of biomass deterioration ○ Low concentration of the algal cake
Flotation Could be described as ‘inverted’ sedimentation in which gas bubbles are produced and provide the lifting force for the particles.	○ Feasible for large scale applications ○ Low cost ○ Low space requirements ○ Short operation times	○ Generally, requires the use of chemical flocculants. ○ Unfeasible for marine microalgae harvesting

Electrical based processes Since microalgae are negatively charged, an electrical field can separate cells (Uduman et al. 2010), either forming a precipitate on the electrodes (electrophoresis) or causing accumulation on the bottom of the vessel (electro-flocculation).	○ Applicable to a wide variety of microalgal species ○ Do not require the addition of chemical flocculant	○ Poorly disseminated ○ High energy and equipment costs
Filtration A dewatering means usually applied after coagulation/flocculation to enhance harvesting efficiency. It works by applying a pressure drop to force the fluid to flow through a membrane.	○ High recovery efficiency ○ Allows the separation of shear sensitive species	○ The possibility of fouling/clogging increases operational costs ○ Membranes should be regularly cleaned ○ Membrane replacement and pumping represent major costs
Centrifugation "Continuous flow centrifuge systems allow sediment-bearing water to be pumped continuously through the bowl assembly, forcing particles to the wall while clarified water passes through the overflow" (Rees, 1991; Dassey and Theegala, 2013).	○ Fast ○ High recovery efficiency ○ Suitable for almost all microalgal species	○ Expensive ○ High energy requirements ○ Suitable only for the recovery of high-value products ○ Possibility of cell damage due to high shear forces

1.1.5 Good management to increase efficiency and reduce the cost of HRAPs

To maximise the efficiency of the microalgae in HRAP system in removing the nutrients, maintaining a healthy culture is important. The first thing to manage is mixing, even mixing throughout the pond for even distribution of nutrients, CO_2, O_2, pH and prevent algae from settling. Mixing algae evenly also provides them with the same amount of light and homogenous temperature, thus maximising productivity. The second thing to manage is algal growth, by keeping the microalgae of interest in the exponential stage for maximum growth to dominate the pond. This could be achieved by regular harvesting of part of the culture. The third factor for successful management is species selection, because of the seasonal and daily (diurnal cycle) variation in environmental conditions. The selected species should have a broad tolerance to variation in temperature, pH and O_2 concentration, and because it is grown in WW it should be able to tolerate contaminants, predators and out compete other microalgae. For this reason, we aimed to bioprospect for algae growing in WWTPs in chapter 3, in a hope to find microalgae that are already adapted to the environmental conditions, WW composition, and could be tolerant to predators (Borowitzka and Moheimani, 2013, pp.133-152). The fourth factor is to reduce the cost of treatment by reducing the cost of harvesting, area and operation by integrating the WW treatment process together with biofuel production from the biomass as a by-product. Costs can be further cut by reducing land area by increasing efficiency by improving microalgae performance as will be discussed in chapter 5.

1.1.6 Algal biomass as a by-product

The algal biomass produced in the WW treatment process can be used as a by-product making the process more economical. The algal biomass could be used as fertilizer, animal feed, and for biofuel production. To use the biomass for animal feed and fertilizers the harvested algal biomass should not have a high concentration of persisting organic pollutants (e.g. organic pesticides), or heavy metals (e.g. cadmium, lead, and mercury) that would be transferred into the animal or contaminate soil (Delrue et al. 2016).

Microalgae biomass can contain ash (5–17%), carbohydrate (18–46%), crude protein (18–46%), and 12–48% high crude lipid (Tibbetts, Milley and Lall, 2015). The biochemical composition of algal biomass varies between species. In addition, microalgae that are grown in both natural and engineered systems can experience a variety of environmental and nutrient limited conditions that affect their growth and cellular composition. Variation in conditions such as temperature or light intensity, and the availability of nutrients such as nitrogen and phosphate can affect cellular composition because of the amount of carbon that is fixed in carbohydrates and lipids is affected by these factors (Juneja, Ceballos and Murthy, 2013). Table 1.2 presents some of these factors and their effects on biomass. In addition to nutrient and environmental factors, the biochemical composition of the biomass could also depend on the algal strain as is the case in *Botryococcus braunii*. In a study to compare the biomass productivity, hydrocarbon and carbohydrate content among 16 strains for *Botryococcus braunii*, the carbohydrate content varied per gram of dry weight biomass from 20–76% (Gouveia et al. 2017). It is important to understand the synergistic interactions between environmental and nutritional factors on different algal strains to develop and improve the productivity of algal systems (Juneja, Ceballos and Murthy, 2013).

Algal biomass as fertilizer

Dried algal biomass can be used as fertilizer as microalgae can recycle nutrients from WW and store it in the algal biomass, this biomass can be used as a sustainable slow-release fertilizer (Benemann, 1979; Mulbry et al. 2005; 2007; Coppens et al. 2016). Using microalgae as an organic fertilizer has several benefits such as preventing nutrient loss because it gradually releases nutrients, nitrogen, phosphorus, and potassium in adequate amounts for plant requirements (Mulbry, Kondrad and Pizarro, 2007; Coppens et al. 2016). Microalgae also contain trace elements and substances that enhance plant growth such as, vitamins, carotenoids, phytohormones, amino acids, and antifungal substances (Spolaore et al. 2006; Coppens et al. 2016). The application of algal biofertilizers has been demonstrated to stimulate plant growth and increase crop yield for cyanobacteria (Tripathi et al. 2008) and macroalgae (Kumari, Kaur and Bhatnagar, 2011).

Table 1.2: Effects of environmental factors and limitation of nitrogen and phosphate on the biochemical composition of algal biomass
Adapted and modified from (Juneja, Ceballos and Murthy, 2013)

Factor	Organism	Variation in condition	Biochemical changes observed	References
Temperature	*Botryccoccus*	Increased from 25 °C to 32°C	Decrease in intracellular lipid content from 22% to 5% wt, accumulation of polysaccharides	(Kalacheva, et al. 2002)
	Chlorella vulgaris	Increased from 20 °C to 38°C	Decreased starch resulting in increased sucrose	(Nakamura and Miyachi, 1982)
		Increased from 10 °C to 38°C	Transformation of L-starch (high molecular weight) to S-starch (low molecular weight); reversible with temperature	(Nakamura and Imamura, 1983)
	Haematococcus pluvialis	Increased from 20 °C to 30 °C	3-fold increase in astaxanthin formation	(Tjahjono et al. 1994)
	Chlorococcum sp.	Increased from 20 °C to 35 °C under nitrogen deprivation	2-fold increase in total carotenoid content	(Liu and Lee, 2000)
	Nitella mucronata Miquel	Increased from 5 °C to 20 °C	Increase in velocity of cytoplasmic streaming	(Raven and Geider, 1988)
Light	*Dunaliella virdis*	Darkness (no light)	Increase in total lipid content, decrease in free fatty acids, alcohol, sterol	(Gordillo et al. 1998)
	Nannochloropsis	Light limited	Increase in lipid content, increase in eicosapentanoic acid (EPA) proportions	(Sukenik et al. 1989)
	Porphyridium cruentum	Red light	Enhanced photosystem II relative to photosystem I and phycobilisome	(Cunningham et al. 1990)
	Chlorella vulgaris	Red light	Increase in sucrose and starch formation	(Miyachi and Kamiya, 1978)
		Blue light	Increase in lipid fraction and alcohol-water insoluble non-carbohydrate fraction	

pH	*Chlamydomonas acidophila s*	pH 4.4	Denaturation of V-lysin	(Visviki and Palladino, 2001)
	Coccochloris peniocystis	pH decreased from 7.0 to 5.0 and 6.0	Decrease in total accumulated carbon and oxygen evolution	(Coleman and Colman, 1981)
Nitrogen	*Nannochloropsis oculata*	75% decrease in nitrogen	Increase in lipid synthesis from 7.90% to 15.31%	(Converti et al. 2009)
	Phaeodactylum tricornutum	Nitrogen limitation	Increase in lipid synthesis, decrease in protein content	(Morris et al. 1974)
	Chlorella vulgaris	75% decrease in Nitrogen	Increase in lipid synthesis from 5.90% to 16.41%	(Converti et al. 2009)
	Haematococcus pluvialis	Nitrogen limitation	Increase in carotenoid formation (13% w/w)	(Borowitzka et al. 1991)
Phosphorus	*Scenedesmus sp.*	50% decrease in phosphorus	Increase in lipid from 23% to 53%	(Xin et al. 2010)
	Ankistrodesmus falcatus	Limitation	Decrease in chl *a* and protein, increase in carbohydrate and lipids	(Kilham et al. 1997) (Healey, 1982) (Healey and Hendzel, 1979)
	Selenastrum minutum	Starvation	Reduced rate of respiration, decreased photosynthetic CO_2 fixation	(Theodorou, et al. 1991)

Phosphorus is an essential nutrient in fertilizers and there are increasing concerns about a future phosphorus scarcity and the sustainability modern agriculture because phosphate rock is not a renewable resource. It has been estimated that phosphate reserves will be completely depleted in around 60-130 years (Steen, 1998; Cooper and Carliell-Marquet, 2013). In 2011, the U.S. Geological Survey (USGS) estimated that phosphate rock reserves could last for 370 years at current extraction rates (Jasinski, 2011). The UK is depending on the imported phosphorus, with net imports ca. 113.5 kt of phosphorus in 2009. Imported fertilisers represent more than half of total phosphorus imports (56%). In the same year, aproximately 55.0 kt of phosphorus was received by WWTPs, around 22.5 kt was recycled in agriculture as sludge from the WWTPs. That application rate was ca. 5X higher than the rate of uptake for crops, raising a new challenge for effective recycling of phosphorus (Cooper and Carliell-Marquet, 2013), it could be washed out by the rain to the water bodies again. Recycling phosphorus from the WWTPs as algal biomass as slow release organic fertilizers can help reduce the stress on phosphorus resources and protect water resources too.

Algal biomass for animal feed

Another by-product of algal biomass is that it could be used as feed for animals ranging from aquaculture to pets and farm animals. Many nutritional and toxicological assessments have demonstrated that algal biomass can be used as a valuable feed supplement, or replace conventional protein sources such as soybean meal and fish meal (Becker, 2007). It has been reported that 30% of world algal production was used for animal feed (Becker, 2004, pp.312-351). Around 30-40% of the production cost for aquaculture is contributed by the cost of microalgal production (Borowitzka, 1997). The benefits of using microalgae as feed for animals is that they are a natural food source, they can be used as a sole nutrient or as food additives for enhancing the colour of the salmon fish, and the yellow colour of broiler skin and egg yolk. They are a source of natural vitamins, minerals and essential fatty acids. The microalgae strains that are used in aquaculture need to be nontoxic and easily cultured strains, with suitable size and shape for ingestion and rich with high quality nutrients pigments or highly unsaturated fatty acid such as eicosapentaenoic acid (EPA), and a digestible cell wall. Some of the species used are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Phaeodactylum* and *Arthrospira* (Spolaore et al. 2006).

In the case of microalgae grown in WW, depending on the microalgal strains that dominate, dried microalgal biomass can be used as feedstock for high value products such as the pigment phycocyanin that is easily extracted microalgal biomass (Griffiths, 2016), carotenoids (Safafar et al. 2015, 2016), and omega 3 (Zhou et al. 2012 and Delrue et al. 2016). It is important to mention that the productivity of the above-mentioned molecules from a microalgal biomass grown in WW is likely to be low because the microalgae need specific conditions to maximise their productivity such as optimum temperature and medium. Another important issue that could hamper the production of animal feed product from WW grown microalgae is the strict regulations issued by the food, cosmetics and pharmaceutical industries (Spolaore et al. 2006).

Algal biomass feed stock for biofuel

The search for environmentally friendly and sustainable energy sources has become a priority due to the harmful effects of greenhouse gas emissions, decreased fossil fuel reserves, and increased energy demand, which is expected to increase by 30% by 2040 (Hannon et al. 2010; IEA, 2016). The world's population is estimated to reach 9.1 billion by 2050 (FAO, 2009), and an increase of the world's real income coupled with higher standards of living adds more pressure to energy resources (Hannon et al. 2010; Jones and Mayfield, 2012). As mentioned above the biochemical composition of algal biomass varies with species and is influenced by different environmental conditions and in an open pond depends on the dominant species. Different types of biofuel may be produced from algal biomass such as biodiesel, bioethanol, biohydrogen, biogas and bio oil (Demirbas and Demirbas, 2010, pp.97-138; Jones and Mayfield, 2012).

If the produced microalgal biomass is rich in lipids it can be used to produce biodiesel, and many species of algae have high lipid storage (Chisti, 2007; Griffiths and Harrison, 2009; Jones and Mayfield, 2012). To produce biodiesel from the algal biomass, the lipids need to be extracted and then subjected to transesterification, and this will increase the cost of production (Hannon et al. 2010). Biodiesel from microalgae is still not economically competitive with petroleum fuel. The cost of a barrel of algae- derived fuel is estimated at US$300–2600 (Chisti, 2007; Hannon et al. 2010; Alabi, Bibeau and Tampier, 2009), compared with $55 for crude oil (Oil-price.net, 2017), this cost is estimated for algal biomass that is not grown in WW.

Bioethanol can be produced from algae that accumulate high levels of polysaccharides. The algal biomass characterised by a low percentage of lignin and hemicellulose as compared to a lignocellulosic plant (Harun, Danquah and Forde, 2010). Some macroalgae such as Ceylon moss (*Gelidium amansii*) have a significant amount of sugars and could be fermented for bioethanol production (Wi et al. 2009). Macroalgae look similar to land plants but they have little lignin crosslinking molecules in their cellulose. Their structures are supported by the buoyancy that allows for upright growth in the absence of the lignin (John et al. 2011). Some species of the microalgae genera like *Chlorella*, *Dunaliella*, *Chlamydomonas* and *Scenedesmus* are known to contain a large amount (>50% dry weight) of starch and glycogen, useful as raw materials for ethanol production (Ueda et al. 1996). A main challenge to produce bioethanol from the algal biomass is the cost of starch/cellulose depolymerising enzymes for the algal biomass pre-treatment increases the cost of bioethanol production by several folds (John et al. 2011).

Biogas is another type of biofuel that can be produced from algal biomass that has been subjected to anaerobic fermentation. The productivity of biogas varies between species, and it depends on how efficiently the cells are degraded and on the presence of inhibitors for the methanogenic archaea (Mussgnug et al. 2010). A limitation for biogas production from microalgae is that the digesters need to be heated and the area and infrastructure requirements for the production are the same for the amount of energy that can be obtained from algal biodiesel (Collet et al. 2011; Jones and Mayfield, 2012).

Biocrude oil is a viscous, energy dense, dark coloured liquid that can be produced from algal biomass by hydrothermal liquefaction (HTL). In the HTL process the whole wet microalgal biomass (proteins, carbohydrates, lipids, and algaenans) is thermochemically converted under high temperature and pressure to biocrude oil, gas and solid as by-products. HTL conditions need temperatures of 250–375 °C, and 10-20 MPa of pressure (Barreiro et al. 2013). It has an advantage that since it converts the whole algal biomass it does not require a high lipid content strain, and fast growing microalgae (high biomass production) will be suitable (Barreiro et al. 2013), it has been used to convert algal biomass grown in WW into biocrude oil (Zhou et al. 2013). The main disadvantage of biocrude oil is that it has a high content of nitrogen 5–7%, which will lead to high NO_x emission rates from combustion. There are several questions that need to be answered for this technology, some regarding reaction pathways and kinetics. In addition, in open pond grown microalgae for WW treatment, it can be difficult to ensure constant properties and composition of the biomass over time since biomass impurities and inhomogeneity needs to be handled carefully to avoid poisoning catalyst active sites or plugging the reactor (Barreiro et al. 2013).

1.2 Concepts of species and delimitation

Species is an essential category of biological organisation. There have been endless debates over an all-encompassing definition for the species and the criteria applied to delimit species (Hey, 2006 and Leliaert et al. 2014), and phycologists are no exception to this (John and Maggs, 1997, pp. 83-107; Mann, 1999, 2010 and Leliaert et al. 2014). The biological species concept proposes that a species is a population of interbreeding organisms. This concept could not be applied to many coccoid green algae that reproduce only asexually. To overcome this limitation, the morphological species concept was adopted but it has been criticised for being too subjective (Krienitz and Bock, 2012). The morphology of microalgae varies under different environmental conditions and leads to uncertainty in algae identification and classification (Luo et al. 2006; Krienitz and Bock, 2012; Darienko et al. 2015). In the meantime, an ultrastructure concept has been used that is based on the anatomy ultrastructure of the flagella and cytokinesis during mitosis (Krienitz and Bock, 2012). The phylogenetic species concept (Cracraft, 1989): ‘species is an irreducible (basal), cluster of organisms diagnosable distinct from other such clusters, and within which there is a parental pattern of ancestry and descent’.

The phylogenetic concept is built on using genetic markers such as 18S rDNA and mitochondrial *cytochrome c oxidase* 1 (*cox1*) for DNA barcoding, which is a method of species identification according to DNA sequence similarity compared to a sequence database of a *priori* defined species (Hebert, Cywinska and Ball, 2003; Leliaert et al. 2014). The chosen marker affects the number of species recognised. On the other hand, the DNA taxonomy method is a DNA-based species delimitation, which describes species boundaries depending on sequence data (Tautz et al. 2003; Leliaert et al. 2014). The species could be delineated by changes in the sequence(s) composition of the gene(s), their chosen threshold of genetic divergence for a sequence being used to recognise the species level of the organism. For microalgae, where species level identification is particularly difficult, applying a polyphasic approach in which the morphological, ultrastructure, ecophysiological and molecular phylogenetic approaches have been suggested (Pröschold and Leliaert, 2007; Krienitz and Bock, 2012).

1.2.1 Phylogenetic markers used for species delimitation in algae

Algae are a phylogenetically diverse group, this makes the application of a single universal marker used to delineate the species and/or for bar coding is very difficult. Different markers for different algal groups are used to delineate their species (Leliaert et al. 2014). Some of the main markers are currently applied for species delineated and/DNA barcoding for some algal groups are presented in Table 1.3. However, the resolution of these markers may not be good enough to distinguish between closely related species in some clades (e.g. Mattio and Payri, 2010; Zardi et al. 2011). The phylogenetic marker should be easy to amplify and have sufficient variation within and between species. The marker can be located in the nuclear, mitochondrial and chloroplast DNA (Table 1.3).

1.3 Methods for improving microalgae

Algae are a diverse group, that ranges from the prokaryotic cyanobacteria to multicellular eukaryotic macroalgae (Guiry, 2012). They can inhibit freshwater and marine environments. Microalgae can grow autotrophically with cost effective and simple cultivation methods. Numerous algae are generally regarded as safe (GRAS) for industrial applications (Gangl et al. 2015). As it was mentioned earlier they act a source of natural food and feed additive, cosmetics, pigments, fatty acids and feed stock for biofuel (Hallmann, 2007). Because of the aforementioned criteria, recently important developments have been achieved for individual species improvement and for the community improvement.

Table 1.3: Markers applied in DNA-based species delimitation and/or barcoding in some algal groups. Adapted and modified from (Leliaert et al. 2014).

Algal group	Marker plastid	 mitochondrial	 nuclear	References
Green algae	***tufA***, *rbcL*		SSU rDNA, LSU rDNA, rDNA ITS	Verbruggen et al. 2007; Leliaert et al. 2009; Hall et al. 2010; Luo et al. 2010; Saunders and Kucera, 2010; Škaloud and Peksa, 2010; Fucikova et al. 2011; Rindi et al. 2011; Škaloud and Rindi, 2013; Subirana et al. 2013
Red algae	*rbcL*, RuBisCO spacer	***cox1***, *cox2-3* spacer	Phycoerythrin, elongation factor, LSU rDNA	Robba et al. 2006; Sherwood et al. 2008, 2010; Le Gall and Saunders, 2010; Kucera and Saunders, 2012; Tan et al. 2012; Janouškovec et al. 2013; Payo et al. 2013
Brown algae	*psbA*, *rbc*L, RuBisCO spacer *psaA*,	***cox1***, *cox3*	rDNA ITS	Lane et al. 2007; McDevit and Saunders, 2009; Mattio and Payri, 2010; Peters et al. 2010; Tronholm et al. 2010; Silberfeld et al. 2013
Diatoms	*rbc*L	*cox1*	SSU rDNA, LSU rDNA, rDNA ITS	Amato et al. 2007; Evans et al. 2007; Vanelslander et al. 2009; Mann et al. 2010; Moniz and Kaczmarska, 2010; Hamsher et al. 2011; Kermarrec et al. 2013

Markers in bold are recommended barcode markers, it should be mentioned that for none of the groups an agreement has been achieved.
*rbc*L, codes for ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit
tufA, encoding protein synthesis elongation factor Tu
atpB, code for ATPase beta-subunit gene
cox 2-3 spacer**,** *cytochrome oxidase* 2-cyto*chrome oxidase* 3 intergenic spacer
psaA, code for Photosystem I P700 chlorophyll a apoprotein A1
psbA, code for Photosystem II protein D1
RuBisCO spacer, Ribulose-1,5-bisphosphate carboxylase/oxygenase spacer
ITS, Internal transcribed spacer
SSU, small ribosomal subunit
LSU, large ribosomal subunit

1.3.1 Improving microalgal species by genetic engineering

The cells of the microalgae naturally contain a wide range of high value compounds that can be enhanced such as phycocyanin from *Arthrospira platensis* (Querques et al. 2015), Docosahexaenoic acid (DHA) from *Pavlova lutheri*, eicosapentaenoic acid (EPA) from *Nannochloropsis gaditana* (Ryckebosch et al. 2014; Gangl et al. 2015) and lipids as triacylglycerides (TAGs) for biodiesel production (Chisti, 2007; Scott et al. 2010). Microalgae can be used as cell factories too for the production of other compounds and proteins such as antibodies, hormones and vaccines (Hallmann, 2007; Gong et al. 2011). There are some benefits of using microalgae as cell factories, they can double their biomass within and less than 24 hours, thus, a short period of time is required for large scale production and for the creation of new transgenic line (Schmidt, 2004; Mayfield et al. 2007). The gene that codes for recombinant proteins can be expressed from the chloroplast or the nuclear genome (León-Bañares, 2004), and the eukaryotic microalgal cells have a post-translational modification pathways, that allow for the production and secretion of glycosylated proteins (Hempel and Maier, 2012; Lauersen, et al. 2013a; Lauersen, et al. 2013b). Many transgenic microalgae can be grown in enclosed bioreactors autotrophically or heterotrophically reducing the risk of escaping to the environment (Gangl et al. 2015).

Recently, algal genome data became available for eukaryotic microalgae; around 28 genomes has been fully sequenced and 4 are in progress (Scaife et al. 2015). Whilst, around 44 genomes were sequenced for cyanobacteria (Peters et al. 2013, pp.37-75). In addition, the algal genetic toolkits were developed such as DNA delivery methods, selection markers, promotors, untranslated regions and reports made it easier to develop transformation protocols for a number of microalgae (Gangl et al. 2015; Mussgnug, 2015). More than 40 different microalgae species have been successfully genetically modified such as *Dunaliella salina*, *Haematococcus pluvialis* and *Chlorella vulgaris*, and the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Gangl et al. 2015).

The ability to use microalgal cells as factories for the production of recombinant proteins has been demonstrated. For example, monoclonal antibodies (complex proteins used for treating human diseases) against anthrax protective antigen 83 (PA83) was expressed in the chloroplast of *C. reinhardtii* (Mayfield, Franklin and Lerner, 2003). Monoclonal antibody against Hepatitis B virus surface protein has been produced in the endoplasmic reticulum of *Phaeodactylum tricornutum* (Hempel et al. 2011; Hempel and Maier, 2012). Immunotoxins which are antibodies coupled to toxins of eukaryotes were produced in the chloroplast of *C. reinhardtii* by (Tran et al. 2013) and the industrial enzyme Xylanase 1 was transformed into the nucleus of *C. reinhardtii* and expressed (Rasala et al. 2012). Only one case is reported for large scale recombinant protein production, that is the milk amyloid A (MAA) was produced in *C. reinhardtii* and grown in three bags 100 L in size in greenhouse conditions (Gangl et al. 2015; Gimpel et al. 2015). Although, genetic engineering of microalgae is a powerful tool for microalgae manipulation, it still has some constraints, it needs knowledge about the gene(s) or the genome of the microalgae and its' methods of manipulation, moreover, obtaining regulatory approval for genetically modified organisms can be an obstacle (Gangl et al. 2015).

1.3.2 Improving microalgal species by random mutagenesis

Random mutagenesis is a relatively easy method that produces a large number of random mutant collections. Random mutagenesis can be produced by chemicals such as ethyl methanesulfonate (EMS), which induces random point mutations by substitution of thymine instead of cytosine opposite to alkylated guanines (Montelone, 1998). In addition, random mutagenesis can be generated by physical mutagenesis such as, the X rays that cause double strand DNA breakages by α-particles, or by ultra violet light (UV) that disturb the DNA structure by the formation of adjacent pyrimidines dimer (Montelone,1998). Another way for generating random mutagenesis is by random DNA insertion, in which a random exogenous DNA introduce in a different location in the nuclear genome. DNA insertion mutation has an advantage of that the sequence can be used to identify the site of the mutation in the nuclear genome (Galvan et al. 2007) for reverse genetic studies, that is knockout the gene to identifying the phenotype (Mussgnug, 2015).

Random mutagenesis was applied to improve the yield of the high value products in microalgae. The UV light was applied to increase the EPA and DHA content in *Pavlova lutheri* (SMBA 60). A mutant strain was obtained *Pavlova lutheri* (II#2) characterized by an increase in the fatty acids content, around 32.8% of EPA and 32.9% of DHA compared to the control strain (Meireles, Guedes and Malcata, 2003). Random mutagenesis was used to increase the lutein production in *Chlorella sorokiniana* by the mutagen N-methyl-

N′-nitro-nitrosoguanidine (Cordero et al. 2011), and to improve lipid productivity and TAGs content in *Nannochloropsis oceanica* IMET1 by heavy-ion irradiation mutagenesis (Ma et al. 2013). Hence, the random mutagenesis can happen in random and multiple places, therefore it is important to consider and investigate the presence of other undesirable side effects (Melis, 2009).

1.3.3 Improving microalgae community by directed evolution

In nature, when microalgae are grown in large scale in open ponds, and if they are grown in WW as well, they would live within a community of organisms and be subjected to the variation of environmental conditions. This community can be manipulated to enhance the productivity (biomass) and crop protection from pathogens and predators as a result of competitive exclusion principle, that is in a diverse culture of organisms, the resources are occupied and this will increase the possibility to competitively exclude the invaders (Kazamia et al. 2014; Nalley, Stockenreiter and Litchman, 2014; Smith and Crews, 2014; Gangl et al. 2015). The resilience of the community (the ability to recover after a disturbance) and the stability of the community (to minimize the fluctuation in the population in spite of a disturbance) can be enhanced too by the manipulation of the community (Nalley, Stockenreiter and Litchman, 2014; Gangl et al. 2015).

One approach to manipulate the community is by directed evolution, in which artificial selection is used to create efficient co-cultures. This selection is based on the ability of the organisms to live and work together for many generations under specific conditions to achieve specific aim from the mixed community (Gangl et al. 2015). For example, water samples from different surface waters that contain algae were collected, and cultured in CO_2 rich environment with the absence of the nitrogen during the light period and in the dark period they were cultured in a nitrogen rich conditions, the repeated cycling between these two conditions over many generations selected organisms that able to produce and store the energy compounds such as starch and lipid to be able to assimilate nitrogen during the dark periods (Mooij et al. 2013). Direct evolution could be applied to the selection of algae community that is resistance to extreme conditions such as high salts concentration and pH (Gangl et al. 2015).

1.4 Overall Aim

The aim of the work reported in this thesis was to develop a generic method to isolate, evaluate and improve locally adapted microalgal species suitable for insertion into large-scale High Rate Algal Pond (HRAP) systems for the sustainable treatment of final effluent wastewater. To achieve sustainability, the HRAP system would need to remove excess nutrients in WW to avoid eutrophication at relatively low capital and operational costs. The overall cost of the system would be mitigated further by maximizing the value of algal biomass produced as a by-product of system operation. To establish such generic method of isolation, evaluation and improvement, the initial bioprospecting component of the species isolation phase was conducted in two geographies, the UK and Jordan, with both very different climates and levels of water availability/scarcity.

The objectives of this study were:

- (Chapter 3) To isolate and identify microalgae native to WWTPs in the UK and Jordan that are presumably adapted to the local environments, and detect determine the species complexity between the Jordan and UK isolates.
- (Chapter 4) Evaluate the microalgae isolates against criteria-based on potential for successful insertion into a HRAP system: growth in WW, efficiency in removing nutrients, and ability to self-flocculate and settle under gravity.
- (Chapter 5) Study the possibility to improve performance in a HRAP system specifically aimed at phosphorus removal by overexpression of Phosphorus Starvation Response 1 (*PSR1*) in the microalgae.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Unless otherwise stated, chemicals used during this study were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., Dorset SP8 4XT, UK). WW nutrients test Spectroquant® Kits (nitrate, ammonium, total nitrogen, phosphate, total phosphorus) are listed in Table 2.9 were purchased from Spectroquant (Spectroquant ®, Merck, Germany).

2.1.2 Media and WW

Different types of media were used for growing microalgal strains, depending on the purpose of experiment and algal species. For microalgae two media were used: Bold's basal medium (BBM) and tris acetate phosphate (TAP) medium compositions are listed in Table 2.2. In many parts of this research WW was used for growing microalgae, the characteristics of which are listed in Table 2.4

Bold's Basal Medium (BBM)

BBM is a nitrate-based medium used as a nutrient rich medium for sample enrichment in bioprospecting experiments and used to grow and maintain the obtained indigenous microalgae isolates. The recipe to prepare 1 L is described in Table 2.1. The final volume is made up to 1 L with deionized water (DIH_2O). Final pH was adjusted to 6.6 with NaOH and was sterilised by autoclaving.

Tris Acetate Phosphate (TAP)

TAP is an ammonium-based medium used for growing the *Chlamydomonas* strains. TAP components are listed in Table 2.2 for preparing 1 L. The pH was adjusted to 7.0 with glacial acetic acid. The final volume was to 1 L with DIH_2O and sterilised by autoclaving. Modifications in TAP medium have been made depending on the purpose of the experiment. TA medium is same as TAP medium, but the phosphate buffer II solution was replaced with 1.5 mM KCl. Another modification on TAP medium included the replacement of phosphate buffer II solution with 100 μM α-D-glucose-1-phosphate disodium salt (Shimogawara et al. 1999; Wykoff et al. 1999). Furthermore, the phosphate concentration was reduced 100X less to around 10 μM phosphate in the low-Pi-TAP (Bajhaiya et al. 2016).

Table 2.1: BBM composition (Bold, 1949; Andersen, 2005, p.437)

Component	Stock Solution ($g{\cdot}L^{-1}dH2\ O$)	Quantity Used (mL)	Concentration in Final Medium (mM)
Macronutrients			
$NaNO_3$	25.00	10	2.94
$CaCl_2{\cdot}2H_2O$	2.50	10	0.170
$MgSO_4{\cdot}7H_2O$	7.50	10	0.304
K_2HPO_4	7.50	10	0.431
KH_2PO_4	17.50	10	1.29
NaCl	2.5	10	0.428
Alkaline EDTA solution		1	
EDTA	50.00		0.171
KOH	31.00		0.553
Acidified iron solution		1	
$FeSO_4{\cdot}7H_2O$	4.98		0.0179
H_2SO_4		1	
Boron solution		1	
H_3BO_3	11.42		0.185
Trace metals solution		1	
$ZnSO_4{\cdot}7H_2O$	8.82		0.0307
$MnCl_2{\cdot}4H_2O$	1.44		7.28×10^{-3}
MoO_3	0.71		4.93×10^{-3}
$CuSO_4{\cdot}5H_2O$	1.57		6.29×10^{-3}
$Co(NO_3)_2{\cdot}6H_2O$	0.49		1.68×10^{-3}

Table 2.2: TAP medium preparation according to Gorman and Levine, 1965; and Chlamydomonas resource centre https://www.chlamycollection.org)

TAP medium	Stock preparation	Quantity used (mL)	Final concentration in 1 L (mM)
Tris base (1M)	**121.14 $g{\cdot}L^{-1}$**	**20**	**20**
Phosphate Buffer II	**for 0.1L**	**1.0**	**1**
K_2HPO_4	10.8 g		0.6
KH_2PO_4	5.6 g		0.4
Solution A	**for 0.5 L**	**10.0**	
NH_4Cl	20 g		0.748
$MgSO_4{\cdot}7H_2O$	5 g		0.083
$CaCl_2{\cdot}2H_2O$	2.5 g		.041
Hutner's trace elements	**for 1L**	**1.0**	
$Na_2EDTA{\cdot}2H_2O$	50 g for (250 mL)		0.134
$ZnSO_4{\cdot}7H_2O$	22 g for (100 mL)		0.136
H_3BO_3	11.4 g for (200 mL)		0.184
$MnCl_2{\cdot}4H_2O$	5.06 g for (50 mL)		0.04
$FeSO_4{\cdot}7H_2O$	4.99 g for (50 mL)		0.0329
$CoCl_2{\cdot}6H_2O$	1.61 g for (50 mL)		0.0123
$CuSO_4{\cdot}5H_2O$	1.57 g for (50 mL)		0.01
$(NH_4)6MoO_3$	1.10 g for (50 mL)		$4.44{\times}10^{-3}$ mM
Glacial acetic acid		**≈1.0 to pH 7.0**	

<u>Luria-Bertani (LB) and super optimal broth with catabolite repression (SOC) medium</u>

LB and SOC medium were used to grow *Escherichia coli*. The components are listed in Table 2.3. After dissolving the components pH was adjusted to 7.0 with 5 M NaOH. The final volume of the solution was adjusted to 1 L with DIH_2O, then sterilised by autoclaving, for SOC medium the sterile glucose was added after sterilisation.

Table 2.3: LB medium components

LB	For 1 L	SOC	For 1 L
Tryptone	10 g	Tryptone	20g
Yeast extract	5 g	Yeast extract	5g
NaCl	10 g	NaCl	0.58g
For solid medium-agar	15 g	KCl	0.19g
		$MgCl_2$	0.95g
		MgSO4	1.2g
		1 M filter sterilised glucose	20 m

WW Characters

WW used in this experiment was collected from the secondary treatment effluent at Somerton WWTP and kept in the cold room until used. Before using the WW, it was filtered through GF/C filter paper (Glass fibre, Grade GF/C, Whatman) and sterilised by bubbling with ozone gas for 15 min followed by filtered air bubbling for 20 min. All UK microalgae isolates were grown in the same batch of Somerton the WW (Table 2.4). For the microalgae isolates obtained from Jordan. Somerton WW was modified to become similar to the average weight of the final effluent from the WWTPs in Jordan in 2013 (Table 2.4), with the addition of 3 M NH_4Cl for ammonium, $NaNO_3$ for nitrate and rock phosphate (10 g/L) for phosphate addition.

Table 2.4: Characteristics of WW used during this research.

Somerton secondary effluent WW					
Test	PO_4-P	NH_4-N	NO_3-N	Tot N	P:N
Average (mg/L)	2.37	0.415	16.8	20	1:8.4
Jordan final effluent WW Year/ 2013					
Test	PO_4-P	NH_4-N	NO_3-N	Tot N	P:N
Weight average (mg/L)	4.3	13.5	12	30	1:7
Modified Somerton secondary effluent WW					
Test	PO_4-P	NH_4-N	NO_3-N	Tot N	P:N
Average (mg/L)	5.77	24.5	25.9	50	1:8.6

*P:N Phosphate nitrogen ratio

2.1.3 Culture conditions

During this study microalgae cultures were incubated at different growth conditions depending on the purpose of the experiment.

- **Growth room optimum growth conditions**
  Samples were incubated in the growth room (Fitotron plant growth room, WEISS Gallenkamp), in the following conditions: light intensity 130-150 $\mu mol \cdot photon \cdot m^{-2} \cdot s^{-1}$, light: dark (L:D) cycle in hours 16:8 and at 22 °C.
- **Samples blooming conditions in the growth room**
  Light intensity 45-55 $\mu mol \cdot photon \cdot m^{-2} \cdot s^{-1}$, L:D 16:8, 22 °C and shaking at 100–120 rpm.
- **Sanyo environmental test chamber conditions**
  Algae were grown in Sanyo MLR-351 environmental test chamber (Sanyo Electric Co., Ltd. 5-5, Keihan-Hondori 2-Chome Moriguchi City, Osaka 570-8677 Japan), at light step setting 3 (LS3) at 50 $\mu mol \cdot photon \cdot m^{-2} \cdot s^{-1}$; L:D 16:8 cycle in hours and 25 °C.
- **Long-term culture growth conditions Sanyo MCO-15AC**
  The plates of microalgal strains were maintained in Sanyo MCO-15AC at 15 °C and supplemented with 3-5 $\mu mol \cdot photon \cdot m^{-2} \cdot s^{-1}$ from a white fluorescent tube.

Light measurements were taken with a LI-COR 250A light meter (LI-COR Environmental St. John's Innovation Centre, Cambridge, CB4 0WS, UK)**.**

2.1.4 Microalgal strains

Scenedesmus obliquus CCAP 267/7 referred as *S. obliquus* was obtained from the Culture Collection of Algae and Protozoa **(**Scottish Marine institute, Argyll PA37 1QA, Scotland, UK). This strain was used as a reference in the WW experiments. The bioprospected strains and the reference were maintained long term by subculturing them in BBM plates and incubating them in a Sanyo MCO-15AC (section 2.1.3)

Chlamydomonas strains

Chlamydomonas strains presented in Table 2.5 were bought from *Chlamydomonas* Resource Centre (University of Minnesota, Minnesota MN 55108, USA, https://www.chlamycollection.org/). *Chlamydomonas* strains were maintained during the research period in tris-acetate-phosphate (TAP), as broth or plates with 1.5% agar in Sanyo environmental test chamber (section 2.1.3) for the short term. All algal strains were maintained for the long term on BBM or TAP agar plates in Sanyo MCO-15AC. long term culture conditions are mentioned in section 2.1.3

Table2.5: Strains of *Chlamydomonas*

Chlamydomonas Strains	Cell wall presence	Growth on nitrate based medium
CC-1010 wild type mt+ [UTEX 90]	Yes	Yes
CC-4267 psr1-1 mt-	Yes	Yes
CC-125 wild type mt+ [137c]	Yes	Yes

- mt here referred to the mating type (either mt+ or mt-)

2.1.5 Software used

- **Nucleotide BLAST**, BLASTN 2.6.0 service provided by the National Centre for Biotechnology Information (Zhang et al. 2000; Morgulis et al. 2008). This software was used to search online for similar sequences for identifying algal species.
- **DNAMAN,** DNAMAN™ Software (Lynnon Biosoft, Quebec, Canada, Version 5.2.9). This program was used for performing multiple alignments and phylogenetic tree construction
- **Primer 3**, Primer 3 Softwear (Koressaar et al. 2007 and Untergasser et al. 2012). Primer 3 was used for designing primers.
- **SnapGene software**, SnapGene Softwear Version 3.2 (from GSL Biotech, IL 60615, USA, available at snapgene.com). Used to build the vector map.
- **SPSS,** IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. All statistical analysis and related graphs were produced using this program.
- **NIS-Elements AR2.30 software,** used for capturing light microscopy photos.

2.2 Methods

2.2.1 Samples collection

Water samples were collected from the UK and Jordan. Samples from the UK were collected from Somerton and Avonmouth WWTPs, date and location of sample collection are presented in (Figure 2.1 A and Table2.6). These two WWTPs are close to Bath and managed by Wessex Water, they treat municipal WW. Avonmouth WWTP is the largest plant managed by Wessex Water, it serves more than one million PE and is located at Avonmouth on the Bristol Channel (Chavez, 2003). Somerton is a smaller WWTP located south of Bath, and serves around 8,000 PE. The final effluent annual average for total nitrogen is around 25 mg/L and the total phosphorus average is about 4 mg/L. Avonmouth WWTP final effluent annual average for total nitrogen is around 45 mg/L and for total phosphorus is likely to be around 7.5 mg/L. It varies depending on the season with higher results in summer (Jack, 2017).

Regarding samples from Jordan five water samples were studied. Three of five are from WWTPs chosen depending on the phosphate and total nitrogen annual average in the final effluent in 2013. Low content was measured in total nitrogen and phosphate around 32 mg/L and 4 mg/L respectively at Al-Fuheis WWTP, while Al-Karak WWTP has a high content of both elements in the final effluent, around 113.6 mg/L for the total nitrogen and 11.5 mg/L for phosphorus, and it is located in the south. The third WWTP is As-Samra, chosen because it is the largest WWTP in Jordan and treats more than 70% of the WW in Jordan. The annual final effluent average for the total nitrogen here is 15.5 mg/L and for phosphate is 3.6 mg/L (Water Authority of Jordan, 2014). Two samples were collected from fresh water resources (Al-Fuheis Spring and King Abdullah Canal). The samples represent different geographical areas with different climates (Figure 2.1B, Table 2.7).

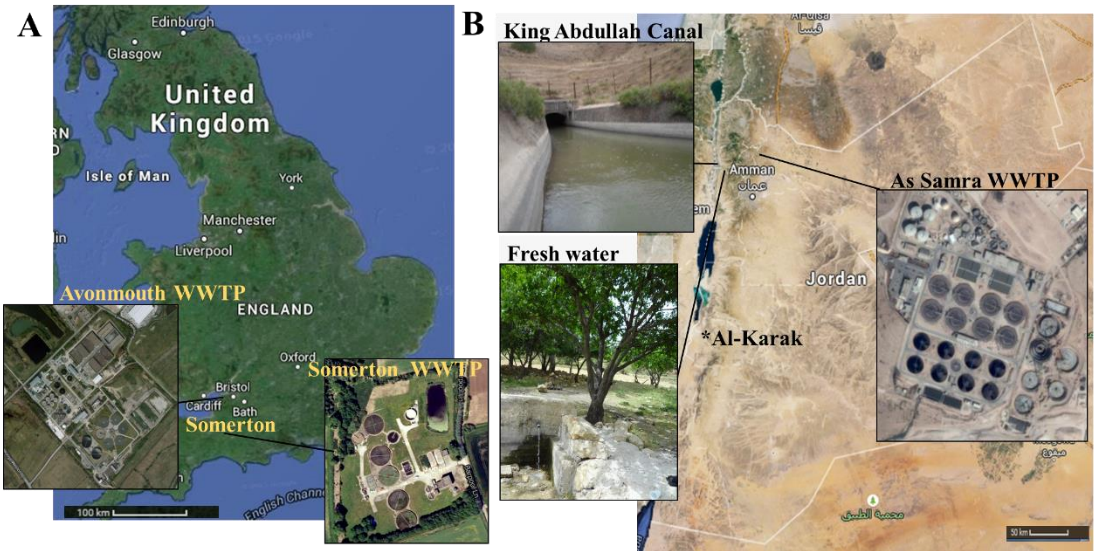

Figure 2.1: Bioprospecting locations in the UK and Jordan. (**A**) Samples were collected from the two WWTPs in the UK shown on the map: Avonmouth WWTP and Somerton WWTP. (**B**) Some of the sampling areas in Jordan, As Samra WWTP, and two fresh water sources: fresh water spring in Al Fuheis City and King Abduallah Canal in the Jordan Valley.

Table 2.6: Details of samples collected the UK

WWTPs	Locations of sampling	Date
Somerton/trickling filters	- Secondary sedimentation tank	23-1-2014
	- Final effluent	
	- Trickling filters	
Avonmouth/activated sludge	- Final effluent	14-2-2014

Table 2.7: Details of samples collected from Jordan

WWTPS		
WWTP / Type of treatment	**Locations of sampling**	**Date**
Al-Karak/activated sludge	- Final effluent	28-4-2014
Al-Fuheis/activated sludge	- Final effluent	29-4-2014
Samra/activated sludge	- Secondary sedimentation tank	5-5-2014
Other Environmental samples		
Place	**Site**	**Date**
King Abdullah Canal (KAC)	El Rayan	6-5-2014
	El Nafaq	6-5-2014
Al-Fuheis	Spring	5-5-2014

Obtaining single colonies

From each location 500 mL of the water body were collected and filtered using GF/C filter paper (pore size 1.2 µm, Whatman). Filter papers were added to 100 mL BBM for enrichment. They were grown until they bloomed (approximately 10 days) in a temperature controlled growth room please see section 2.1.3 for sample blooming conditions. Bloomed samples were serial diluted up to 1×10^{-6} and 150 µL from the diluted samples were spread on BBM plates (1.5% agar). Plates were then incubated in the growth room at optimum growth conditions (Section 2.1.3) until colonies were observed (Figure 2.2).

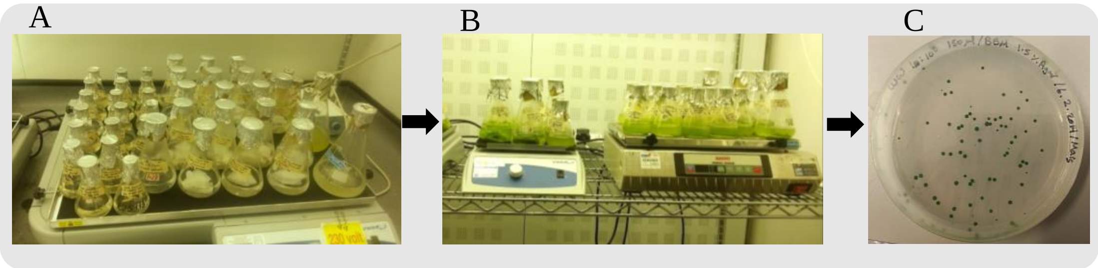

Figure 2.2: Bioprospecting process flow till obtaining single colonies. (A) After filtering the samples through GF/C filter paper, the filter papers were added to 100 mL of BBM for enrichment. (**B**) Samples bloomed after 10 days and their colour turned green. (**C**) After serial dilution and plating on BBM agar plates, single colonies were obtained.

2.2.2 Morphological examination

Agar plates were examined under a dissecting microscope (Nikon SMZ 1500, Nikon Digital Sight DS-U1camera, NIS Elements F2.30 Software). Colonies of green colour but different in size, shape and colour intensity were picked. After examination under the compound light microscope (Nikon Eclipse 90i, Nikon Digital Sight DS-U1camera, NIS-Elements AR2.30 software), cells that were different in shape, size, colour and colonial form were subcultured in 1.5 mL BBM in small glass tubes and cultured in the growth room at optimum conditions (section 2.3) with shaking at 100-120 rpm by Sanyo orbital shaker (MIR-S100). Bloomed microalgae were checked for purity under the microscope, then subcultured on BBM agar plates (1.5 % agar). This single colony subculturing process was repeated 2-3 times to ensure the purity of the algal isolate from any kind of contamination by other microalgae, bacteria or fungus. It was then subjected to DNA isolation.

2.2.3 DNA isolation

For DNA extraction, a single colony was picked from an agar plate and allowed to grow in 5 mL BBM or TAP media in Sanyo environmental test chamber conditions (section 2.1.3) with 250 rpm shaking. DNA isolation was performed according to Doyle and Doyle (1987) with the following modifications: around 10-60 × 10^{-6} cells were collected (variation due to cell growth rate) in a 1.5 mL microcentrifuge tube and homogenised in liquid nitrogen. Then 750 µL of preheated (60 °C) CTAB isolation buffer [2% hexadecyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM ethylenediamine-tetraacetic acid (EDTA), 100 mM Tris-HCl pH 8.0] were added. The samples were incubated at 60 °C for 30 min with gentle mixing from time to time, and they were then purified with the same volume of chloroform and centrifuged at 8160 ×g for 10 min. The upper aqueous phase was transferred into a new clean microcentrifuge tube, the nucleic acids were precipitated by the addition of 2/3 volume of cold isopropanol and kept at −20 °C for 30 min, followed by centrifugation for 15 min at 16000 ×g. The pellet was washed with 500 µL of 80% ethanol and centrifuged for 10 min at 16000 ×g. The pellet was then dried and resuspended in 50 µL of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.2.4 Polymerase Chain Reaction (PCR)

PCR was used for many purposes throughout this research, such as DNA barcoding of microalgae, amplification of a target gene and as a screening tool to confirm the presence of the construct during the transformation process.

A standard PCR reaction was performed in a final volume of 25 µL including: As a template 50–250 ng for genomic DNA or around 10 ng of the plasmid, for the colony PCR the mix was inoculated directly with bacterial cells; each primer 0.4 µM; Dream *Taq* ™ Green PCR Master Mix 2X (Thermo Scientific, Waltham, MA, USA) 12.5 µL. The reaction was run in a Peltier Thermal Cycler (PTC-200, MJ Research, USA) under the following amplification conditions: initial denaturation at 94 °C for 4 min, followed by 40 cycles of 94 °C for 45 s each, annealing for 45 s, extension at 72 °C in a rate of 1 min for 1 kb and a final extension step at 72 °C for 7 min. The annealing temperature (T_a) varies with the primer used. In general, it was chosen as 5 °C below the lowest melting temperature (T_m) for the primers.

In the case of *Chlamydomonas reinhardtii* which has a high GC content (ca. 64% of its genome; Blaby et al. 2014), a modification to the PCR reaction was made to include the addition of dimethyl sulfoxide (DMSO) at a final concentration of 5% to enhance PCR amplification.

High-fidelity PCR was performed according to the the protocol of Q5 High-Fidelity DNA Polymerase from (New England Biolabs Ltd, Herts SG4 0TY, UK). In a total volume of 25 µL containing 50-250 ng genomic DNA, dNTPs 240 µM, from each primer 0.4 µM; Q5 reaction buffer and Q5 high GC enhancer buffer were added at a final concentration of 1X, and 2 units of Q5 High fidelity DNA polymerase. The thermal cycling conditions were: initial denaturation at 98 °C for 3 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at T_a for 30 s and extension at 72 °C for 20-30 s per kb, then a final extension step at 72 °C for 4 min. The reaction was performed in a Peltier Thermal Cycler (PTC-200, MJ Research, USA). The annealing temperature varied depending on the primers used. Manufacturers recommended that the T_a would be 3°C above the T_m of the lower T_m primer.

All the primers used in this research were bought from Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany). Internal primers (18S-I-2JF, 18S-I-2JR, 18S-I-4JF, and 18S-I-4JR) were designed to help in sequencing the long 18SrDNA PCR product from Jo_2 and Jo_4, which were > 2300 bp in length. A set of internal primers. upstream and downstream for the *PSR1* gene were designed and illustrated in Figure 2.3. All primers used are listed in Table 2.8.

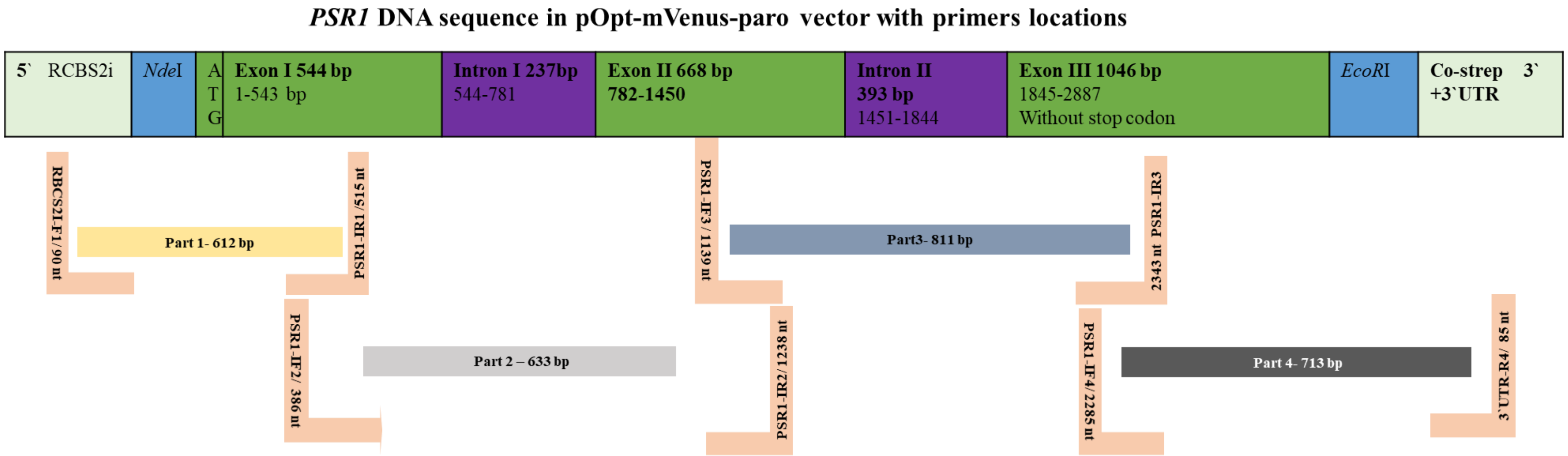

Figure 2.3: Whole *PSR1* gene in the pOpt-mVenus-Paro vector illustrates the primers used with their location and the expected fragment length.

Table 2.8: Primers used in this study

Primer name	Target sequence Amplicon length	Sequence 5`-3`	Reference
DNA Bar coding primers			
16S1N 16S2N	18S rDNA Amplicon ≈ 1700 bp	TCCTGCCAGTAGTCATATGC TGATCCTCT/CGCAGGTTCAC	Grzebyk et al. 1998
ITS-F ITS-R	ITS1-5.8S-ITS2 Amplicon 600 bp-1500 bp	GAAGTCGTAACAAGGTTTCC TCCTGGTTAGTTTCTTTTCC	Timmins et al. 2009
18S-I-2JF 18S-I-2JR	Internal region of 18S rDNA for Jo-2*	ACAGGGATTCGGCATGGTTA AAGGGCAGGGACGTAATCAA	This study
18S-I-4JF: 18S-I-4JR	internal region of 18S rDNA for Jo-4*	GCTGTTATGCCTGCTAGTCG CCCAACAGGCTCTCATCCAA`	This study
***PSR1* amplification and sequencing primers**			
PSR1F-RS-1 PSR1R-RS-1	Full *PSR1* gene with restriction sites (*Nde*I, forward) and (*EcoR*I, reverse) Amplicon = 2889 bp	GGTATAC<u>CATATG</u>GACAAAGCT GAACGCGCTGC TAGACG<u>GAATTC</u>TGGCTCCACT CGCTGCCGCTTTG	This study
PSR1F-OB-1 PSR1R-OB-1	Full PSR1 gene with 120 bp upstream the start codon and 78 bp downstream the stop codon Amplicon = 3106 bp	CGCCGCTTGTCAGTTTCAA TCTGTGGCTTGGCTGGTTAG	This study
PSR1-IR1 PSR1-IF2 PSR1-IR2 PSR1-IF3 PSR1-IR3 PSR1-IF4	Internal primers for PSR1 gene used for sequencing*	CGGCTGCATGAAGTACTGC CCGCATCTACTAGCACCGA GACCGTGCCCCTAGAGATG CGGTGCGCTAATCTCCCC GTGTGCCTGCCCTGCTAG GAGCTTGTTGTGGCGGGG	This study
Primers located in the vector			
RBCS2i-F1	Used for sequencing the full length *PSR1* in the vector*	TCCTCTGTCGCTGTCTCAAG	This study
PSR1-OEC-F PSR1-IR1	For confirming the construct presence in the transformed Chlamydomonas Amplicon = 1168 bp	GCTGAGGCTTGACATGATTGGT GCG CGGCTGCATGAAGTACTGC	This study
Paro-R-F Paro-R-R	Paromomycin resistance gene Amplicon = 755 bp	AGTGGGTTGTTGTGGAGGAT GAAGAACTCGTCCAACAGCC	This study
AR-OEC_R	Used to detecting the empty vector with Paro-R-F Amplicon =1140	TTGTGTGGAATTGTGAGCGG	This study

*These internal primers did not work in pairs there is no amplicon produced
The primers were designed using Primer 3 software (Koressaar et al. 2007 and Untergasser et al. 2012).

2.2.5 Plasmid characters and isolation

The nuclear expression vector pOpt_mVenus_Paro (Lauersen, Kruse and Mussgnug, 2015) was selected according to many characteristics such as strong constitutive promoters and an antibiotic selection marker and reporter (Figure 2.4). It was obtained from the Chlamydomonas centre. The pOpt_mVenus_Paro vector has been constructed for nuclear gene expression by Lauersen and coleagues in 2015. The pOpt_mVenus_Paro vector has the following regulatory elements:

- Constitutive promotors: *Chlamydomonas reinhardtii* heat shock 70A (*HSP70A*) promoter fused with RuBisCO small subunit 2 (*RBCS2*) promoter with RuBisCO small subunit intron 1 (*i1*; abbreviated *HSP70A-RBCS2-i1*; Figure 2.4). This promoter structure has been reported to be efficient for nuclear transgene expression in *Chlamydomonas reinhardtii* (Lauersen et al. 2013).
- The RBCS2 3′ untranslated region (3′UTR) in addition to the regulatory regions.
- A selection marker, aminoglycoside 3`-phosphotransferase gene (*aph*) from *Streptomyces rimosus* (*aphVIII)* was inserted in a separate expression cassette. The *aphVIII* gene confers resistance for paromomycin, kanamycin, and neomycin. Paromomycin was used to select transformed colonies (Sizova, Fuhrmann and Hegemann, 2001).
- Reporters to help selection. The expressed protein of interest is tagged with a StrepII affinity tag (*StrepII-TAA*), a synthetic peptide consisting of eight amino acids and a stop codon (WSHPQFEK*). The florecent protein m_Venus is used as reporter for subcellular targeting studies in *C. reinhardtii*. Since its signal proved to have minimal spectral interfereance with the background of the chlorophyll pigment. In this vector each element was enclosed by unique restriction sites for easier modification of the vector (Lauersen, Kruse and Mussgnug, 2015).

For plasmid isolation, 5 mL of sterile LB medium with 150 µg/mL a final concentration of the antibiotic carbenicillin. The LB media was inoculated with a single colony of *E. coli* (DH5α). The culture was placed in a shaking incubator overnight at 37 °C, 200 rpm. The cells were harvested by centrifugation at 3230 ×g. The plasmid was isolated from the cell pellet by QIAprep Spin Miniprep Kit (Qiagen Ltd., UK) according to manufacturer instructions.

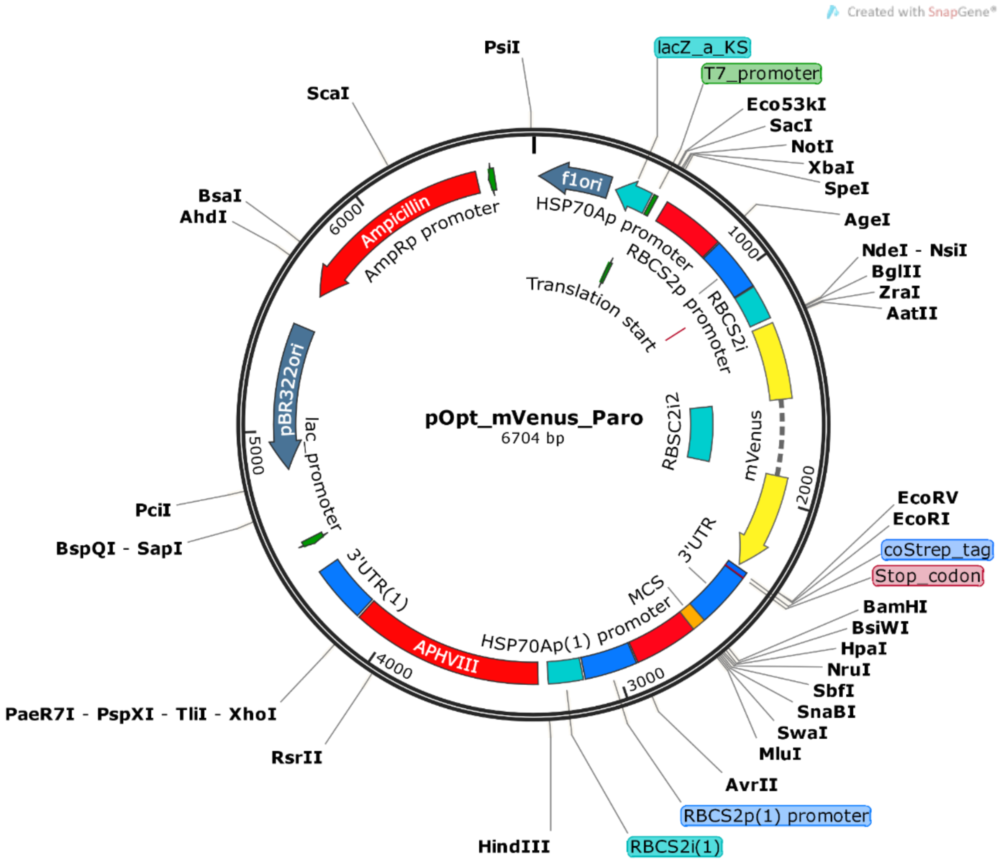

Figure 2.4: pOpt_mVenus_Paro vector map created by SnapGene Software Version 3.2.

2.2.6 Plasmid manpulation

To prepare the construct containing the gene of interest (*PSR1*), the vector pOpt_mVenus_Paro and the *PSR1* gene were double digested with *Nde*I and *Eco*RI-HF (New England Biolabs Ltd., Herts SG4 0TY, UK)The vector was double digested to remove the mVenus sequence (1067 bp) from the vector to be replaced with the gene of interest. The *RSR1* gene was double digested with the same enzymes to produce a complementary end to the vector to facilitate the ligation process. The digestion reactions were performed according to the manufacturer's instructions. One microgram of the substrate (*PSR1* gene or the vector pOpt_mVenus_Paro) and ten units of each enzyme in a total reaction volume of 50 µL at 37 °C for 3 hours. The digestion reaction was then stopped by heating at 65 °C for 20 minutes. The digested DNA fragments were analysed by agarose gel electrophoresis and purified.

The ligation reaction was performed using T4 DNA ligase (Promega Corporation, WI 53711, USA). In a total volume of 10 µL with a 1:1 molar ratio of vector:insert, around

100 ng of the vector and 51.8 ng of insert were incubated overnight at 4 °C. the sequence of the constructs was verified by sequencing.

2.2.7 Agarose gel electrophoresis

The genomic DNA and PCR products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% (w/v) sucrose) to a final concentration of 1X. The genomic DNA samples were analysed on 0.7% and the PCR products on 1% agarose gel in Tris-Acetate-EDTA buffer (1X TAE, 40 mM Tris base, 20 mM Acetic acid, and 1 mM EDTA pH 8) stained with 0.5 µg/mL ethidium bromide to visualise the PCR product. Samples were run simultaneously with 100 bp DNA ladder (Invitrogen™ Life Technologies, CA, USA), or 1 kb DNA Step Ladder (Promega Corporation, WI 53711, USA) for product size estimation. The concentration and the quality of nucleic acids (genomic DNA, PCR product and plasmid) were quantified using a NanoVue UV spectrophotometer (GE Healthcare) to measure the absorbance at 260, 280 and 230 nm.

2.2.8 Purification of PCR products, digestion reactions and plasmid

The purification of PCR products, digestion reactions and plasmids for sequencing or cloning purposes was done by Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, WI 53711, USA) according to the manufacturer`s instructions.

2.2.9 Transformation of *E. coli*

To preserve and amplify the plasmid it was transformed by a heat shock method into α-Select Chemically Competent Cells from (Bioline Reagents Ltd. UK). α-Select Competent Cells have the selection marker *lac*Z gene which provides α-complementation of the β-galactosidase gene for blue and white colony screening. α-Select Competent Cells contain *rec*A1 and *end*A1 markers to decrease the recombination and increase the insert stabilty.

Transformation was done according to the manufacturer’s instructions. From the ligation reaction 5 µL were added to 50 µL of α-Select Competent Cells mixed and incubated on ice for 30 min. They were then subjected to heat shock at 42 °C for 45 s then placed on ice for 2 min. To help the cells recover, 950 µL of SOC medium were added and incubated for 1 h at 37 °C. Two hundred microliters of the culture were plated on LB agar plate with

150 µg/mL of carbenicilin for selection, the plates were incubated overnight at 37 °C. To confirm the successful transformation and the presence of the insert, a colony PCR was performed using RBCS2i-F1and PSR1R-RS (Table 2.8), and a double digestion with *NdeI* and *EcoRI* enzymes. Two plasmids from some of the positive colonies were then sent for sequencing for verification. Strains of *E. coli* were stored in 15% glycerol at −80 °C for long-term storage.

2.2.10 Sequencing and sequence analysis

Samples were prepared and sent for sequencing as follows: around 15 µL of 10 ng/µL of purified PCR product and (50–100) ng/µL of purified plasmid were sent for sequencing (Eurofins Genomics, Ebersberg, Germany). Analysis of the sequences was done using DNAMAN Software Version 5.2.9 (Lynnon Biosoft, Quebec, Canada) and BLASTN 2.6.0 service provided by the National Center for Biotechnology Information (NCBI; Zhang et al. 2000; Morgulis et al. 2008)

2.2.11 Antibiotic sensitivity test

Antibiotic sensitivity for the *C. reinhardtii* strains (CC-1010, CC-125 and CC-4267) and Av_12 were tested by monitoring their growth in a spot test in agar plates and in liquid culture. In the spot test, the microalgae strains were grown in TAP medium, while they are in the log phase they were diluted to a concentration of 1×10^6 cell/mL and 10 µL (approximately 10,000 cells) were blotted in TAP media plates with different paromomycin concentrations (5, 10, 100 and 1000) mg/L (Garcia-Echauri and Cardineau, 2015). The plates were incubated at Sanyo environmental test chamber conditions (section 2.1.3) for 7 days. The growth of microalgae strains was studied in liquid TAP medium with paromomycin addition. Two strains: CC-1010 and Av-12 were seeded in liquid 10 mL TAP medium at cell density $0.6–0.7 \times 10^6$ cell/mL with a final paromomycin concentration of 10 mg/L and 100 mg/L in triplicate, and a control culture without the addition of antibiotic. The cultures were grown for seven days in Sanyo environmental test chamber conditions (section 2.1.3) with mixing by stirring at 200 rpm (2 mag magnetic motion, MIX 15 eco, Muenchen, German). The growth was monitored by daily cell count using Guava easyCyte™ flow cytometer (Millipore, Hayward, CA 94545, USA).

2.2.12 Transformation of the microalgae using electroporation

It was reported that linear DNA increases the chance of the insertion of multiple copies in a single locus and leads to fewer rearrangments or deletions (Cerutti et al. 1997 ; Kindle, 1998). So, to enhance the possibilty of the integration of the plasmid into the nuclear genome, the plasmid was linearised by digestion with *Xba*I enzyme (Promega Corporation, WI 53711 USA). In which 10 µg of the plasmid was digested in a total reaction volume of 100 µL. For each µg of the plasmid, 3 units of enzyme were used and the reaction mix was incubated at 37 °C for 4 h. The digestion mix was then purified by Wizard® SV Gel and PCR Clean-Up System and ready to be used for transformation.

Chlamydomonas strains the wild type (CC-1010_WT) and *psr1* mutant (CC-4267_mt), in addition to Av_12 were transformed by electroporation following the protocol from Invitrogen using GeneArt max efficiency reagent and Gene Pulser X cell electroporation system from Bio-Rad laboratories (Bio-Rad Laboratories Ltd., Hertfordshire HP2 7DX, UK). The transformation was performed according to the manufacturer's instructions. Microalgae strains were subcultured in 200 mL of TAP medium until they reached the early exponential phase and reached a cell density of 1-2 × 10^6 cell/mL and no more than 3 × 10^6 cell/mL. The cells were then harvested by centrifugation for 3230 ×g for 5 min followed by washing twice with 10 mL GeneArt® MAX Efficiency® Transformation Reagent. After that they were resuspended at a cell density of 2 × 10^8 cell/mL, and 250 µL microalgal cells were mixed with 3 µg linearized plasmid in an ice-cold 0.2 cm cuvette and incubated at 4 °C for 5 minutes. This was followed by electroporation using the Gene Pulser X cell electroporation system at 500 V, 50 µF and 800 Ω, incubated on the bench for 15 min and then transferred to 10 mL TAP-40 mM sucrose medium without antibiotic and allowed to recover for 14–16 h in the dark at 22 °C without mixing. Cells were then harvested, resuspended in 300 µL TAP medium, plated in two TAP plates with 10 mg/L paromomycin for *C. reinhardtii* and 300 mg/ L paromomycin for Av_12 and kept in the growth rooms at optimum conditions for 5–7 days.

For Av_12 many modifications were applied to this protocol such as changing the voltage, the resistance, DNA concentration and the buffer was replaced by TAP, TAP-40 mM sucrose and osmosis solution contains 0.2 M mannitol, 0.2 M sorbitol and 10% glycerol (Guo et al. 2013). In addition multiple squar wave pulses were applied too, in which a multiple pulses are delivered to the cells, the number, the voltage, the duration and the intervalses between pulses can be manipulated to deliver the DNA to the cells. A published electroporation protocol for *S. obliquus* (Guo et al. 2013) was applied too. In which 800 µL of *S. obliquus* at cell density of 1×10^8 cell/mL resuspended in the osmosis solution mentioned above mixed with 40 µg of linearized plasmid DNA and salmon sperm DNA, around 400 µL were subjected to 2000 V for 3ms.

For each experiment, a negative control was prepared by electroporating microalgal cells without plasmid DNA. In addition to the negative control, algal cells were transformed with the vector alone to see if it would give the same effect (phenotype) that the vector with insert gives.

Selection of microalgae transformed colonies

To confirm the presence of the construct in the obtained colonies, random colonies were picked and subcultured for at least two constitutive times on TAP plates with 10 mg/L paromomycin to ensure the stability of the transformation. They were then inoculated into 5 mL TAP medium without antibiotic and grown for 7-10 days before being subjected to DNA isolation and PCR to detect the presence of the construct.

2.2.13 Putative transformed microalgae screening

Screening the putative *PSR1* overexpression lines

Six putative *PSR1* overexpression lines generated from CC-1010_WT were confirmed to have the *PSR1* expression construct. These six lines were screened beside the CC-1010_WT and CC-4267_mt, for how efficiently they grew in low-Pi-TAP (section 2.1.2). The samples that grew the best will be then chosen for further phenotyping analysis. This experiment was done in transparent, flat bottomed 96-well microplates. From exponential phase cultures, were resuspended in 10 µM TAP at a cell density of 0.5×10^6 cell/mL, and 250 uL of the culture loaded in each well. Each selected putative overexpression line had three biological replicates and 6 technical replicates with an average of 18 wells for each line. The plates were incubated in Sanyo environmental chamber conditions (section 2.1.3) without shaking. The absorbance of the samples was measured at 750 nm by a microplate reader (Modulus microplate, Turner BioSystems, CA 94085 USA).

Complementation test

This test was done to study the ability of the construct to complement the *psr1* mutation in CC-4267_mt by the ability of producing extra cellular phosphatase. Ten lines of CC_4267 were confirmed to have the *PSR1* expression construct. These lines were grown in modified TAP medium replacing the phosphate buffer by organic phosphate, that is 100 μM of glucose monophosphate (α-D-glucose-1-phosphate disodium salt) this medium was used previously to study the complementation of the *psr1* mutant (Shimogawara et al. 1999; Wykoff et al. 1999). If the construct was able to complement the mutation and produce extracellular phosphatase the line will grow in similar rate to the wild type CC-1010_WT. If it failed to do that the cells would not be able to use the phosphate in the medium and this will affect their growth. This complementation experiment was conducted using 96-well microplates as in the screening experiment. The transformed cell lines were resuspended in modified TAP medium described above in a final concentration of 0.5×10^6 cell/mL. In each well 250 μL of the culture were loaded. Each selected line had 6 technical replicates. The plates were incubated in Sanyo environmental chamber conditions (section 2.1.3) without shaking. A daily measurement for the absorbance of the samples at 750 nm was taken using a microplate reader (Modulus microplate).

2.2.14 Microalgal culture setting conditions

Growing microalgae in wastewater conditions

To study the performance of the microalgae isolates in WW for growth, nutrients removal and biomass production. The first step is preparing seeding cultures as the following, from a single colony, a culture was established in 10 mL of BBM, checked for purity, then scaled up to 100–150 mL of BBM culture. To reduce the lag phase period, the algae isolates were pre-grown in autoclaved WW for acclimatization (for almost 7 days), till they reached a cell density enough for seeding new cultures for nutrients removal experiments.

The experiment was set in 500 mL Duran bottles assembled with silicon tubes for supplying and exhausting of 2.5% CO_2-enriched air (Figure 2.5). Each algal isolate was then seeded at *ca*. 1.0×10^6 cells/mL in triplicate with a final volume of 400 mL of WW per bottle. The samples were incubated in growth room optimum growth conditions (section 2.1.3) mixed by stirring at 350 rpm, and supplied with 2.5% CO_2-enriched air. The cultures were grown for 10 days until they reached the early stationary phase.

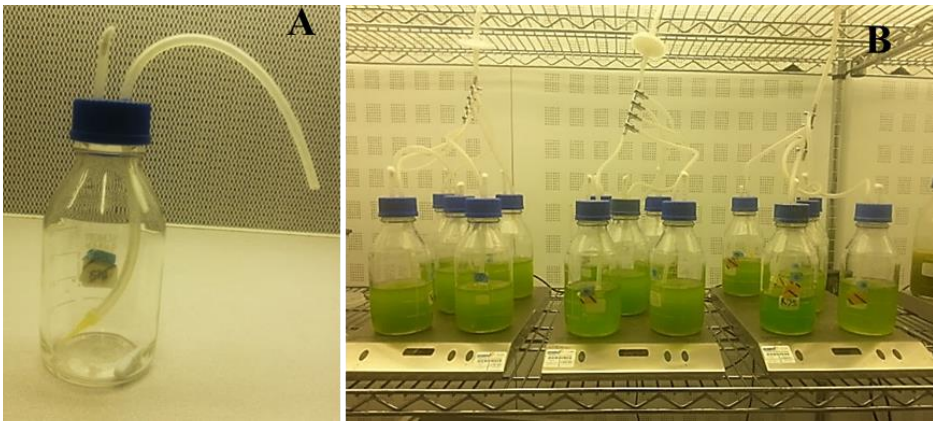

Figure 2.5: Batch experiment setting. (A) Duran bottles (500 mL) modified as for growing algae. (B) Experiments were set and supplied with CO_2 and mixed by magnetic stirrer.

Phenotyping experiment conditions

The purpose of this experiment was to characterise the phenotypes of the putative overexpression lines, regarding growth rate, specific phosphate removal and phosphorus content in the cells. Five microalgae strains were studied, three of them were as control strains: CC-4267_mt, CC-1010 WT and the wild type strain that was transformed with the empty vector (without the insert) CC-1010_vector_only. The remaining two were transformed colonies confirmed to have the construct (pOpt_mVenus_Paro-PSR1) CC-1010_A-6 and CC-1010_B-2.

The strains were grown in TAP medium with two different phosphate concentrations: a normal TAP with 1 mM phosphate and Low-P_i-TAP with 10 µM phosphate (section 2.1.2). Before seeding the cultures, the algal pellets were washed twice with TA medium, a TAP without phosphate (section 2.1.2), to ensure that there was no phosphate transported with the microalgae from the media. They were then seeded at a cell density around 1×10^6 cell/mL in a final volume of 400 mL, and grown in 500 mL bottles for 10 days. The growth conditions were: light intensity 70 µmol photon· $m^{-2} \cdot s^{-1}$, L:D 18:6 cycle in hours, at 22 °C and mixing at 250 rpm. During the experiment, a daily measurement was done for the dry weight, cell count, and OD to assess growth of the microalgae, phosphate in the media and total phosphorus in the algal cells was measured to assess their efficiency to remove phosphate. Phosphorus content was evaluated at two different time points, the first at phosphate depletion from the low-P_i-TAP medium and the second at the end of the experiment. During the experiment, a PCR was done to check the stability of the construct in the growing transformed algae.

2.2.15 Assessment of microalgae growth

Microalgae growth was monitored by tracking the change in optical density, cell count or dry weight. It is essential to obtain a good representative sample to minimise sampling error, therefore it is important to mix the culture well before taking the sample and to sonicate the samples to break up clumps if necessary. This is difficult because the cultures tend to stick to surfaces or each other.

- Cell count was conducted using a Guava easyCyte™ flow cytometer (Millipore, Hayward, CA 94545, USA), from the sample 1 mL was used for cell count. Since the range of cells in a sample that the flow cytometer is able to count is 1×10^5-1×10^6 cell/mL, the sample was diluted. Before taking the cell count the samples were sonicated in an ultrasonic bath (Fisher Scientific, FB15046, Elma) for 5-10 min to break up clumps.
- Optical density (OD): The OD of a 1 mL sample of algae culture was measured at 750 nm by a Spectroquant® Pharo 300 spectrophotometer (Merck Millipore, Darmstadt, Germany). At 750 nm, chlorophyll interference is at a minimum and this method is not recommended when microalgae cultures tend to clump, and the absorbance is also affected by variations in cell size and pigment content (Borowitzka and Moheimani, 2013).

- Dry weight (DW): 15 mL of sample was spun down at 3230 ×g for 5 min and the supernatant was removed (used for the chemical tests). The pellet was resuspended in Milli-Q water and transferred to a 1.5 mL microcentrifuge tube, centrifuged at 16000 ×g for 1 min and the supernatant was remove. The pellet was then freeze dried (Freezdryer Modylyo, Edwards, Britain; Vacuum pump RV8, Edwards, England; Speed Vac concentrator, Savant, Stratch Scientific, London). The dry weight of the sample was measured on a Mettler UMT2, Switzerland balance.
- Specific growth rate and generation time: Growth rate for the algae was calculated during the exponential phase based on dry weight. The growth rate and generation time were calculated using Equations 1 and 2 respectively according to Anderson, 2005.

Growth rate equation (Equation 1):

$$\mu = \frac{\ln\left(\frac{DW_t}{DW_i}\right)}{\Delta t}$$

The specific growth rate is represented by μ. Units for μ are always expressed per unit time (t^{1}); DW_i, the dry weight at the beginning of a time interval; DW_t the dry weight at the end of the time interval. The time period is represented by Δt ($\Delta t = t_f - t_i$), chosen between two points at the exponential phase.

Generation time (doubling time) equation (Equation 2):

$$T_g = \frac{0.6931}{\mu}$$

T_g is doubling time for the culture, expressed in the same units of time as μ**,** which is a day in this study.

2.2.16 Nutrient tests

Nutrient quantification for phosphate, total phosphorus, ammonium, nitrate and total nitrogen was carried out every day until they were completely consumed by the algae. All the tests were done using Spectroquant® Kits (Table 2.9) (Spectroquant ®, Merck, Germany) according to manufacturer instructions. From each sample 15 mL were taken at each time point, centrifuged at 3230 ×g for 10 min, and the supernatant was used for the phosphate, ammonium, nitrate and total nitrogen tests. For the total phosphorus test from algal cells, 5 mL from the culture were collected in a separate 15 mL conical tube and the supernatant was discarded, then the pellet was washed with 1 mM EDTA to remove any externally bounded phosphate and then washed with DIH_2O water. Cells were then collected by centrifugation at 3230 ×g for 30 min. A pre-treatment for the pellet was done before conducting the total phosphorus test. Algal cells were digested in reagent P-1K from the phosphate cell test kit by heating the cells to 120 °C for 30 min in a preheated thermoreactor Spectroquant TR620 (Spectroquant ®, Merck, Germany).

Phosphate uptake evaluation:

To assess how fast microalgae can remove phosphate from WW, equation 3 was used.

Equation 3

$$\text{Specific phosphate removal rate/day} = \frac{|\Delta PO_4 - P|}{(\Delta t * \overline{DW})}$$

$|\Delta PO_4 - P|$ is the absolute value of the change in the amount of phosphate in the WW between two points in the exponential phase, $\Delta PO_4 - P = (PO_4 - P_f) - (PO_4 - P_i)$, $PO_4 - P_f$, the phosphate concentration at the point before the level of phosphate dropped to undetectable level, $PO_4 - P_i$, the phosphate concentration (mg/L) at the initial time point, Δt ($\Delta t = t_f - t_i$), the time period between the two points chosen for the phosphate measurements, t_i, the initial time point and t_f, is the final time. $\overline{DW}$ is the average dry weight during the time period.

To calculate the total phosphorus (P) content in the cells. Equation 4 was used:

$$\frac{P(\mu g)}{10^6 \text{ cells}} = \frac{\text{P content per sample}}{\text{cell numbers}} = \frac{P\left(\frac{mg}{L}\right) * \text{Volume of sample (L)} * 1000}{\text{Cell density}\left(\frac{cell}{mL}\right) * \text{algal volume (mL)} * 10^{-6}}$$

To calculate the percentage of total phosphorus in the algal biomass. Equation 5 was used:

$$\% \text{ P (wt/wt)} = \frac{\text{Total phosphors content per sample } (\mu g)}{\text{DW } (\mu g)} * 100\%$$

$$= \frac{P\left(\frac{mg}{mL}\right) * \text{sample volume (L)} * 1000}{DW\left(\frac{\mu g}{mL}\right) * \text{algal volume (mL)}} * 100\%$$

Nitrogen uptake evaluation:

To evaluate which algae is more efficient in removing total nitrogen from WW, Equation 6 was used:

$$\text{Specific nitrogen removal rate/day} = \frac{|\Delta N|}{(\Delta t * \overline{DW})}$$

$|\Delta N|$, $|\Delta N = N_f - N_i|$ is the absolute value of the change in the amount of phosphate measured in the WW between two time points located in the exponential phase. N_i is the total nitrogen concentration (mg/L) at the initial point, while N_f is the total nitrogen concentration at the final point before the level of total nitrogen dropped to an undetectable level. Δt is the time period between the two points chosen for the total nitrogen measurements ($\Delta t = t_f - t_i$), t_i is the initial time point and t_f is the final time. $\overline{DW}$ is the average dry weight during the time period. Equation 3 and equation 6 were designed with the help of Prof. David Leak (Department of Biology and Biochemistry, University of Bath).

Table 2.9: Nutrient test kits and relevant methods

Test	Principle of the method
Phosphate test **0.0025–0.5000 mg/L PO_4-P** Cat.No. 1.14848.0002	Orthophosphate ions react with molybdate ions in sulfuric solution to form molybdophosphoric acid, which was reduced to phosphomolybdenum blue (PMB) using ascorbic acid, which can be determined photometrically.
Total phosphorus from algal cells **0.5–25 mg/L PO_4-P** Cat.No. 1.14543.0001 **0.5–25 mg/L PO_4-P** Cat.No. 1.14729.0001	In sulfuric solution orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue (PMB) that is determined photometrically. pretreated sample (as mentioned above)
Ammonium test **0.05–3.00 mg/L NH_4-N** Cat No. 1.14752.0002 **2.0–75.0 mg/L NH_4-N** Cat No. 1.00683.0001	In strong alkaline solution, ammonium ions are converted to ammonia. In the presence of a chlorinating agent, they react to form monochloramine, which in turn reacts with thymol in the low range ammonium test (and substituted phenol in high range ammonium test) to form a blue indophenol derivative, which can be determined photometrically.
Nitrate test **0.5–20 mg/L NO_3-N** Cat No. 1.14773.0001	Nitrate ions react with benzoic acid derivatives in a concentrated sulfuric acid to produce a red nitro compound, which is measured photometrically.
Total Nitrogen test- **0.5–15 mg/L N** Cat No. 1.14537.0001 **10–150 mg/L N** Cat No.1.14763.0001	According to Koroleff's method, the organic and inorganic nitrogen compounds were converted into nitrate by the addition of oxidising agents and incubation in at 120 °C for 1 h in a thermoreactor. In the low range total nitrogen test (0.5–15 mg/L), nitrate reacts with benzoic acid derivatives in concentrated sulfuric acid to form a red nitro compound. While for the total nitrogen test range (10–50 mg/L) nitrate reacts with 2,6-di-methylphenol (DMP) to form 4-nitro-2,6-dimethylphenol for photometric determination.

2.2.17 Algal Settleability

A simple experiment has been carried out to assess how fast a microalgae isolate could be harvested by gravity when grow for WW treatment. The sludge volume index (SVI) is a test used in WWTPs to measure the tendency of the activated sludge solids to settle during a sedimentation or thickening process. It is performed by filling an Imhoff cone or a 1L cylinder with mixed liquor and allowed to settle for half an hour.

This simple test was used to evaluate the settleability of the microalgae isolates. Microalgae were grown in a filter and sterile WW in growth room optimum growth conditions (section 2.1.3), with mixing by magnetic stirrer at 350 rpm and 2.5% CO_2-enriched air supplementation, until they reached mid-exponential phase. They were then diluted until they had a similar cell number in a final volume of 400 mL. The test was performed in a 500 mL glass bottle. The height of the settled sludge was measured at different time points (0, 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 24 hours). The height was then converted into a volume using the formula of cylinder volume ($V = \pi r^2 \times h$) to calculate the settled sludge volume. The concentration of suspended solids was measured as a DW concentration as described in section 2.2.15. The SVI was then calculated for the microalgae isolates after 1, 8 and 24 hours using Equation 7:

$$\text{Sludge volume index (SVI)} \left(\frac{\text{mL}}{\text{g}}\right) = \frac{\frac{\text{settled sludge volume}}{\text{sample volume}} \left(\frac{\text{mL}}{\text{L}}\right) * 1000(\text{mg/g})}{\text{suspended solids concentration (mg/L)}}$$

Equation 7 was adapted from the web page of water program sacramento state (http://www.owp.csus.edu/glossary/sludge-volume-index.php)

To add more information and avoid some of the previous methods' limitations, the cell count was measured using a Guava easyCyte™ flow cytometer as described above, taken at the same time points, at a fixed point at ca. 1 cm depth from the top.

Equation 8:

$$\text{Percentage of settled cells} = \frac{\text{cell count (t = 0)} - \text{cell count (t = x)}}{\text{cell count (t = 0)}} * 100\%$$

2.2.18 Statistical analysis and bioinformatics

Unless stated, all statistical analysis and graphs were produced in IBM SPSS Statistic, version 22.

Some bioinformatics work was performed during this research. For sequence identification, Nucleotide BLAST was used. Multiple alignments and the phylogenetic tree were performed using DNAMAN. Primer design was conducted using Primer3, and the vector map was constructed using SnapGene.

Chapter 3

Microalgae isolation and identification for wastewater treatment

3.1 Introduction

3.1.1 Bioprospecting for the selection of algal species

Microalgae are a diverse group of organisms, to date 35,000 species of microalgae have been described, and the actual number of species is much higher. They can inhabit most environments on Earth (Borowitzka, 2013, pp. 77-89). Microalgae are evolutionarily diverse microorganisms, they are rich resources for bioprospecting for microalgal species that can be commercially utilised for the production of bioactive compounds such as cytotoxic, antifungal, and antiviral compounds (Patterson, Larsen and Moore, 1994); the production of organic metabolites like β-carotene and phycocyanin (Borowitzka, 1992); for metabolic conversion such as the bioconversion of progesterone (Pollio et al. 1994); and biofuel production (Stephens et al. 2010) in addition to the WW treatment (Borowitzka, 2013, pp. 77-89).

Growing microalgae in an open pond system for WW treatment means that there is limited control over many environmental factors. So, the microalgae will experience a variation in the environment over daily and annual cycles. For this reason, the selected microalga should be able to tolerate variation in temperature, light, pH and O_2. concentrations (Borowitzka and Moheimani, 2013, pp.133-152). The first step in applying microalgae for any commercial production process is the selection of the microalgal strain (Borowitzka, 2013, pp. 77-89)

The microalgal strain can be obtained from the culture collection or could be isolated from the environment, although it is easier and faster to select and get the strains from culture collections. Only a relatively small part proportion of microalgae species that occur in nature are present in algal culture collections, many species and strains can be isolated from nature (Borowitzka, 2013, pp. 77-89). In addition, to isolate and utilise strains that are native (indigenous) to the environment is advantageous because they can easily adapt to changes in the environment such as diurnal and seasonal variation. This adaption is a competitive criterion essential for open pond systems. Whereas, culture collection strains—because of the continuous sub-culturing to keep them viable—become adapted to the cultivation conditions in the culture collection, and take longer to acclimatise to the environment where they will be applied (Rawat et al. 2013). Many WW indigenous microalgae species have been reported to efficiently grow, remediate nutrients and display high biomass productivity in WW batch cultures. This is because they are adapted to WW and the environmental conditions (Jiménez-pérez et al. 2004; Zhou et al. 2011; Osundeko, Davies and Pittman, 2013).

3.1.2 Climatic factors affect microalgae dominance in the WW

Some of the climatic factors that affect the growth and productivity of microalgae in WW were presented in section 1.1.4, such as the light, temperature and pH, variation in these factors during different seasons also affect the algae population that dominates the WW. In 1996, Canovas et al. reported the seasonal variation of the phytoplankton in HRAPs for WW treatment in the Mediterranean region over two constitutive years. They found that *Chlorella* and *Scenedesmus* genera dominated the ponds. In spring *Scenedesmus* was the major phytoplankton component, whereas, *Chlorella* dominated in winter and autumn reportedly due to lower predation rates and a higher relative growth rate at lower irradiation levels.

A series of experiments reported a seasonal variation in microalgae species in HRAPs that treated WW in New Zealand (Park, Craggs and Shilton, 2011b; Sutherland et al. 2014a). One of these studies aimed to improve species control and harvestability of microalgae from a two pilot-scale HRAPs treating domestic WW in Ruakura Research Centre, New Zealand. Each HRAP had a surface area of 31.8 $m^2$and a total volume of 8 m^3 (Park, Craggs and Shilton, 2011b). Throughout a year of monitoring a total of 13 genera of green algae were found in the WW. The HARP was dominated by four colonial algae that have previously been reported to dominate WW treatments HARP in different parts of the world (García, Hernández-Mariné and Mujeriego, 2000; Park and Craggs, 2010). These were *Pediastrum sp.*, *Desmodesmus sp.*, *Micractinium pusillum*, and *Dictyosphaerium sp.*. During the year, there was a change in the algal species that dominated the HARP between *Micractinium sp.*, *Pediastrum sp.*, *Thalassiosira sp.* and *Dictyosphaerium sp.*, this was attributed to seasonal variation in solar irradiation, temperature which were asserted to play a role in species selection, coexistence and succession in addition to hydraulic time retention. (Park, Craggs and Shilton, 2011b). Recently (Sutherland et al. 2014a) obtained a different result in a year-round performance study of microalgae in a large HRAP system in Christchurch, New Zealand. In this case the 5ha HARPs were dominated all year around by *Micractinium pusillum* Fresenius with an average representation of 90%, with various other species present at low numbers including *Desmodesmus communis*, *Desmodesmus opoliensis*, *Pediastrum boryanum*, *Actinastrum hantzschii*, *Closterium sp*., *Closteriopsis sp*. and *Coelastrum sp.* (Sutherland et al. 2014a)

3.1.3 Climates of sampling sites

When growing microalgae in an open pond system for WW treatment in a large scale, there will be a limited control on the environmental conditions. The variation in the environmental conditions can affect the algal growth and productivity. It is important to obtain microalgae that are adapted (indigenous) to that environment which will increase the efficiency of nutrients removal and biomass production. In this research, it was intended to bioprospect the WWTPs in the UK and Jordan for indigenous microalgae that are adapted to the WWTPs conditions to be applied to the country where it has been isolated from. The climate of the UK is very different from the climate in Jordan and can affect the dominant species in the water bodies.

The UK lies between longitudes 8 °W to 2 °E and latitudes 49 °N and 59 °N. This wide range of latitudes is associated with a mean temperature variation of 4°C from north to south, with the south enjoying warmer conditions during the growing season. The climate in the UK is affected by maritime influence from the west to the east, and the change in altitudes of lands relative to the sea level in addition to the north south latitudes range. The effect of these factors varies between seasons. There are different quarters of climate zoning in the UK including: the north-west areas (Northern Ireland and Cumbria), the summer is cool with temperature means 13-15 °C, the winter is mild with temperature 4-6 °C comparatively to the latitude. The north-east zone (eastern Scotland and north -east England), they have a cool summer and cold winter with temperature mean 3-4 °C. The eastern, south eastern and central lands of England characterized by warm summer and cold winter. The last quarter zone is the south-west areas including Wales and south-west England, this quarter has a warm summer (mean 15-16 °C) and mild winter (mean 5-7 °C; Hopkins, 2008). Table 3.1 provides more details regarding the total area, population, the availability of water and pressure on water for the UK and Jordan.

The first sampling site in the UK was Avonmouth WWTP. Avonmouth city is outer suburb of Bristol (located at 51.501°N 2.699°W), it lies west-north-west to Bristol city centre at the mouth of the River Avon and on the eastern shore of the dredged. The climate information available from the nearest climate station to Avonmouth, which is Filton climate station during the period 1981-2010 were for the annual average of: the maximum temperature average is 4.2 °C, the minimum temperature is 7 °C, days of air frost around 35 days, sunshine hours are 1627 hour, and rainfall is around 802 mm (Met office, 2017a). The second sampling point in the UK was Somerton located at 51.0535°N 2.7361°W and is 45 km south from Bristol and 14 km, the climate information obtained from the nearest climate station to Somerton which is Yeovilton. During the period 1981 to 2010 the annual average from Yeovilton climate station of: the maximum temperature 14.6 °C, the minimum temperature 6.2 °C, days of air frost 53 days, sunshine hours 1563.8 hour, and the rainfall 708.5 mm (Met office, 2017b).

The Hashemite Kingdom of Jordan located in the eastern part of Mediterranean region lies to the east of Jordan River with geographic coordinates in (35 °E-39 °E) longitude and (29 °N-33 °N) latitude. The total population of Jordan is 9.559 million (DOS, 2015a) including more than 1.3 million refugees (DOS, 2015b) Table 3.1. The climate of Jordan varies across different regions. For instance, in Jordan Rift Valley (JRV) along the western border of the country the climate is semitropical, Mediterranean in the highlands, whereas the climate is continental in the eastern desert and plains region. During the period 1985-2014, the mean maximum air temperature in summer (July) varied between 31.5-40 °C while the mean of the minimum temperatures was17.5-21.0 °C. In winter (January), the means of maximum and minimum temperatures were 12.6-20.5 °C and 2.2-9.3 °C, respectively. The largest part of the country -more than 91 %- receives less than 200 mm of rainfall, and only 0.7 % of the country has an annual precipitation of more than 500 mm. The majority of the land (83 %) is desert and desert steppe (Al-ansari et al. 2014). The total renewable water resources (TRWR) cubic meter per inhabitant per year in 2014 was 123.4 m^3/inhab/yr (FAO, 2016a).

Water scarcity (Figure 3.1 A) is assessed by an annualised ratio of water volume per inhabitant. Where the TRWR is below 1700 m^3/inhab/yr the region is considered subject to water stress. If the TRWR drops below 1000 m^3/inhab/yr, the country or region faces water scarcity. When it is below 500 m^3/inhab/yr, as in the case of Jordan, this means, the TRWR indicates that the country is experiencing absolute water scarcity. Figure 3.1 A and B shows the variation in water precipitation and TRWR among different parts of the world including the UK and Jordan (UN, 2014).

Table 3.1: Water resources in the UK and Jordan

	United Kingdom**	Jordan*
Total area (million ha) 2015	24.361	8.932
Total population	64.716 million	9.559***
Long term average annual precipitation (mm/year)	1220	111
Total renewable water resources per capita (m^3/inhab/yr) 2014	2271	123.4
Pressure on total freshwater withdrawal as % of TRWR / year	5.454% in 2012	92.44 % in 2005

*FAO, 2016a, **FAO, 2016b, ***(DOS, 2015a)

Pressure on renewable water resources a very simple indicator for national water scarcity. If the withdrawal % is more than 25 % means the country is water-stressed, more than 60 % is approaching physical water scarcity, more than 75% is facing severe physical water scarcity

http://www.fao.org/nr/water/aquastat/didyouknow/index2.stm

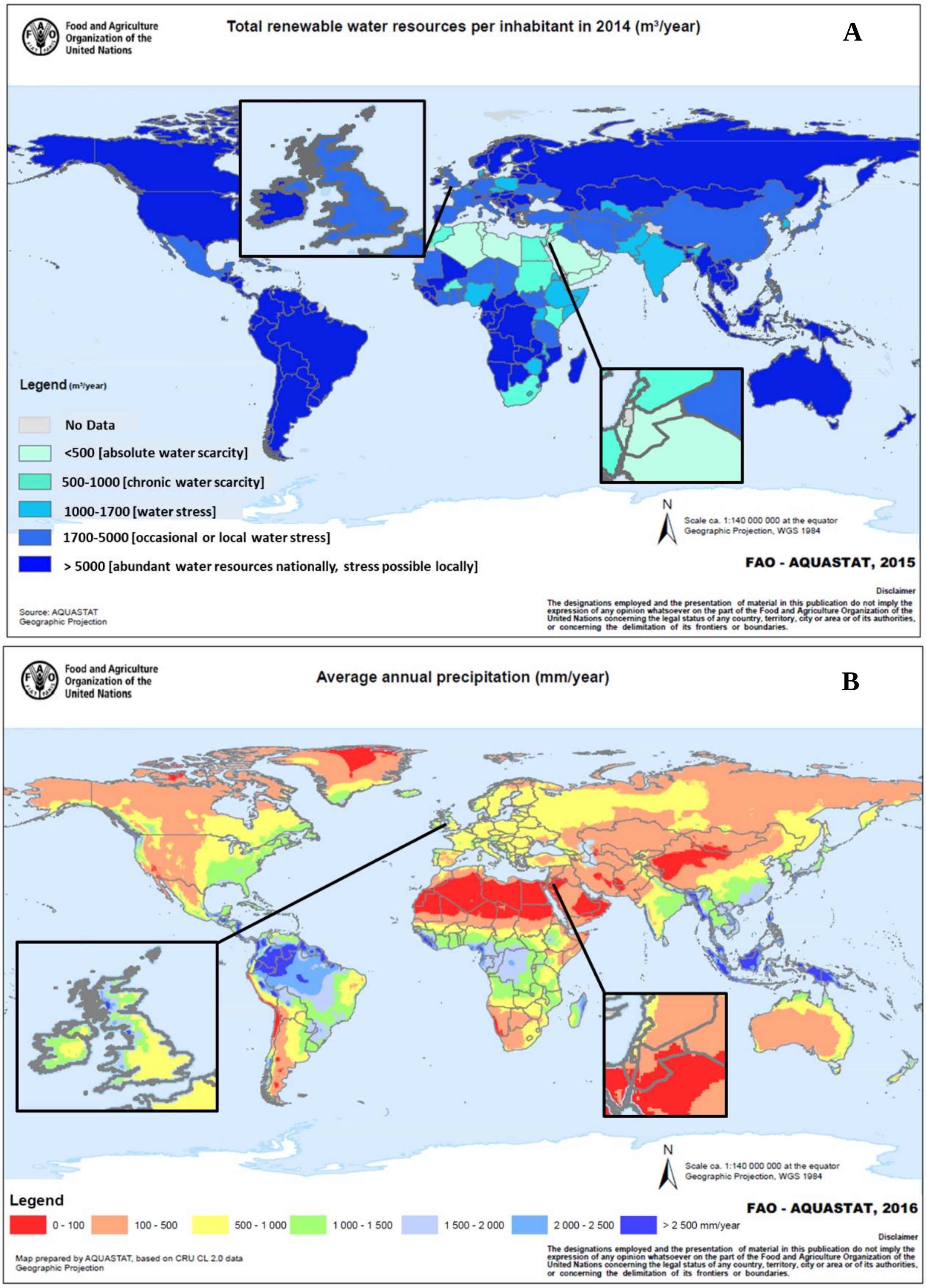

Figure 3.1: Variation in total renewable water resources (TRWR) and annual precipitation among different parts of the world. (A) the total renewable water resources in 2014. **(B)** average annual precipitation in mm/year. Adapted from world map from FAO, 2017a.

3.1.4 Identification of green algae

Green microalgae are documented as growing well in WW stabilization ponds (Palmer, 1974, cited in Palmer 1977) for this reason the bioprospecting process will focus on this group of microalgae. Green algae are conventionally classified according to the variation in their morphology into classes and orders for instance, monadoid (flagellates), coccoids, or filamentous species (Pröschold and Leliaert, 2007, pp.123-153). This morphological method can depend on single or even negative criteria such as the absence of structures such as pyrenoids. Another method of classification using the ultrastructure of the cell, including whether the basal bodies in the flagellar apparatus are clockwise (CW) or counter clockwise (CCW), and the pattern of cell division in mitosis have been considered for identifying algae. More recently, phylogenetic analyses based on the ribosomal gene sequences such as small subunits (SSU) and the Internal Transcribed Spacer (ITS) rDNA confirmed the classification based on the ultrastructure of the cell (Pröschold and Leliaert, 2007, pp.123-153). However, most of the algal genera and orders are polyphyletic and the relationships between several phylogenetic lineages remain ambiguous. It has been suggested that using a polyphasic approach for species and generic characterization. for green algae classification would improve the situation (Pröschold and Leliaert, 2007, pp.123-153; Darienko et al. 2015). Polyphasic approaches consider multiple criteria such as the results of crossing experiments, sporangium autolysin data, ITS rDNA sequences and the secondary structures of SSU, as well as studies of life cycles (Pröschold and Leliaert, 2007, pp.123-153).

Morphological classification

Morphological classification is a classical method to identify algae but it is not necessarily sufficient to provide a classification at the species level for many reasons. Firstly, in some cases the morphology of algae do not correlate well with the phylogenetic position. For example, the coccoid green microalgae often lack characteristic morphological features thus different phylogenetic species may be hidden under a single morphotype (Krienitz and Bock, 2012). Secondly, the morphology of green algae can vary under different environmental conditions, potentially leading to uncertainty in the identification at the species level, particularly when examining a natural sample using the light microscope. Often the spines warts formation and changes in cell wall structure are due to environmental factors. For instance, it has been recorded that grazing affects the morphology of microalgae and causes the formation of bristles in non-bristled *Micractinium sp*.. In *Scenedesmus obliquus* infochemicals released by the rotifer *Brachionus calyciflorus* induces large colony formation (Luo et al. 2006). In another case, *Desmodesmus sp*. reportedly undergo a change in cell wall structure in response to changes in nutrient availability and supplementation (Surek, 2008).

In 2001 Krienitz and his colleagues compared the traditional generic concepts with 18S rDNA sequence for classification of the family Selenastraceae in the green algae phylum Chlorophyta. They concluded that ‘‘the molecular analyses showed that any morphological criterion considered so far is not significant for the systematics of the Selenastraceae on the generic level’’ (Krienitz et al. 2001). Consequently, it has been recommended to re-evaluate the morphological criteria used for traditional classification for each genus. Finally, the morphological characterization could be a time consuming process and require experience (Darienko et al. 2015).

Molecular characterization

According to Krienitz and Bock (2012), molecular characterization appears a reliable method to distinguish organisms and define species and genera in a cost effective and timely manner. DNA barcoding, a method that uses short and polymorphic genetic markers within the nuclear or organelle genomes, has become a popular method to identify organisms to the species level. Barcoding methods are potentially useful to identify the algal isolates. The ideal barcode supposed is a short fragment recognized by universal primers i.e. ones based on highly conserved target sequences thereby enabling easy DNA amplification and should contain a degree of polymorphism to discriminate organisms at the species level (Krienitz and Bock, 2012).

Ribosomal sequences are of the most popular barcodes, as some regions of the gene are highly conserved allowing the design of universal primers, they are present in the genomes of all living organisms, there are multiple copies of those genes in the genome. Until recently, the main source of data to clarify the phylogenetic relationships between green algae taxa (any named group or rank of organisms in a biological classification in which related organisms are classified) was derived the nuclear encoded 18S and 28S rDNA genes (Pröschold and Leliaert, 2007, pp.123-153; Leliaert et al. 2012). More recently, polymorphic sequences termed the (ITS) located between the rDNA genes have become important in the identification of microalga. ITS1 is located between 18S and

5.8S rRNA, while ITS2 is located between the 5.8S and 28S (Figure 3.2). ITS2 is preferred by some scientists because of its sequence variability combined with conserved folding pattern (Buchheim et al. 2011). As the ribosomal operon may contain several versions of ITS2, indels of numerous nucleotides may be present in the less-conserved parts of the different ITS2 versions (Krienitz and Bock, 2012).

The eukaryotic ribosomes are composed of large subunit (LSU) and small subunits (SSU) with four rRNA species. The 28S, the 5.8S and the 5S rRNA species are in the LSU. Whereas, 18SrRNA is found in the SSU. The rRNA species are coded by two genes and transcribed by two different RNA polymerases. The main transcriptional unit, the ribosomal cistron has the transcripts of the 18S, 5.8S and 28S rRNAs and it is transcribed by RNA polymerase I (pol I). The remaining rRNA gene (5S rRNA) is transcribed by RNA polymerase III (pol III) separately from the ribosomal cistron (Mandal, 1984; Paule and White, 2000 cited in Torres-Machorro et al. 2010).

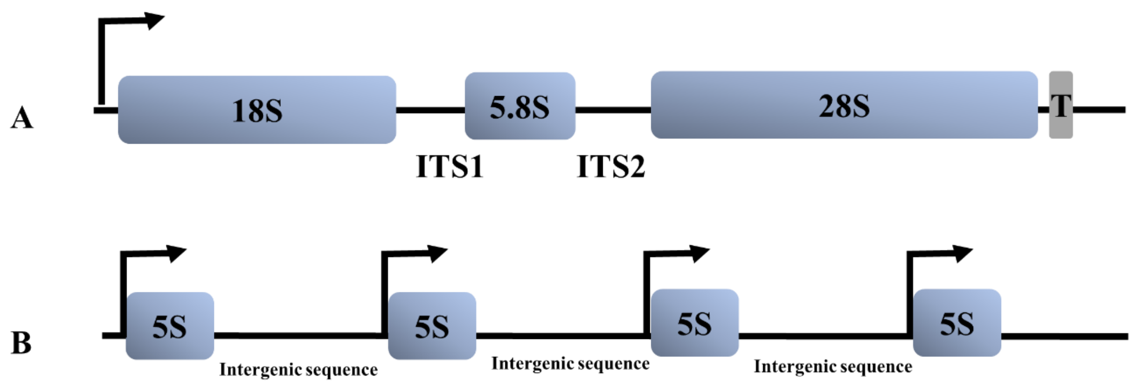

Figure 3.2*: Schematic representation of *Xenopus laevis* rDNA *species* organization.* (A) The arrangement of the nuclear ribosomal cistron, 18S rRNA, 5.8S rRNA and 28S rRNA molecules are transcribed as a single unit and separated by ITS1 and ITS2 regions indicated by the black line, T*;* transcription terminators (T). (**B**) The 5S rDNA in *X. laevis* is organized in tandem repeats that include a coding region (blue box) and an intergenic sequence (black line). The 5S rDNA promoter is internal to the coding region. Arrows represent the transcription start point. Modified from (Torres-Machorro et al. 2010).

3.1.5 Seeding microalgae in open ponds for WW treatment

Applying microalgae for nutrient removal from WW can be done by two strategies. The first is seeding the pond by a well-adapted microalgal strain. The second way is to establish a natural indigenous consortium in the pond (Li et al. 2011). Using a monoculture of algae is required when looking for a specific product from the microalgae, such as a high lipid content, heavy metal removal or production of carotene. This strategy requires a screening process in advance to select the algal strain that is the best in achieving the specific aim. Recently many screening and bioprospecting processes have taken place (Delrue et al. 2016). For instance, 14 strains from the culture collection representing different genera (*Haematococcus*, *Scenedesmus*, *Chlorella* and *Chlamydomonas and Chloroccum*) have been screened to find a robust strain for nutrient removal from WW and accumulate biomass for biodiesel production. The highest net biomass accumulation was achieved by *Chlorella kessleri*, followed by *Chlorella protothecoides* (Li et al. 2011). Many bioprospecting studies have been done for selecting a strain that is efficient in both nutrient removal from WW and high lipid production. One example was in Minnesota in the USA, in which 60 samples were collected from different sampling sites, *Auxenochlorella protothecoides*, *Hindakia sp*. and *Scenedesmus sp*. were among the top performing microalgae (Zhou et al. 2011). After the screening process and before applying it in large scale, the selected microalgal strain has to be tested how it could adapt to the variation in environmental conditions and how it will behave in the WW that is contaminated with different microorganisms (Delrue et al. 2016).

The other strategy to apply microalgae for WW treatment is as consortia, either bacterial-microalgal consortia or multiple microalgal strains consortia. The bacterial-algal consortia have been studied for nutrient removal from WW (De-Bashan et al. 2004; Hernandez, de-Bashan and Bashan, 2006; De-Bashan et al. 2008; Pires et al. 2013). Using the bacterial-algal consortium has some benefits, it has been reported that the growth rate for both the bacteria and the microalgae in the consortium was higher than their growth rate in monoculture. This increase in growth is thought to be due to a potential benefit for the bacteria and microalgae that occur when they are cultivated in WW. Microalgae provide the bacteria with oxygen, which they use for degradation of organic compounds. This process of oxidation will produce CO_2 that will support microalgal growth (Muñoz and Guieysse, 2006; Subashchandrabose et al. 2011; Pires et al. 2013). Furthermore, the bacterial-microalgal consortia can accomplish complicated functions that are hard for a single species to do. The consortia can also tolerate fluctuations in environmental conditions (Brenner, You and Arnold, 2008; Pires et al. 2013). An additional advantage is that bioflocculation of the consortium helps in the harvesting of biomass by gravity sedimentation which reduces the cost of harvesting (Park, Craggs and Shilto, 2011b).

Immobilising bacterial-microalgal consortia in alginate beads of *Chlorella vulgaris*/ *Azospirillum brasilense* and *Chlorella sorokiniana*/*Azospirillum brasilense* has been reported to improve nitrogen and phosphorus removal from municipal WW. The nutrient removal percentages after 6 days for the consortium were 36% phosphorus, 100% ammonium, and 15% nitrate; whereas the monoculture of microalgae removed up to 19% phosphorus, 75% ammonium, and 6% nitrate. This shows that the consortium with bacteria improves the nutrient removal efficiency of microalgae (De-Bashan et al. 2004). Another example of a consortium of different microalgal species was isolated from carpet industry WW (*Chlamydomonas globosa*, *Chlorella minutissima* and *Scenedesmus bijuga*) for biomass production. The mixotrophic algal consortium *Chlamydomonas globose*/*Chlorella minutissima* for poultry litter extract and *Scenedesmus bijuga*/*Chlorella minutissima* for carpet WW were recommended for renewable biomass for biofuel production (Bhatnagar et al. 2011; Pires et al. 2013).

When applying a synthetic consortium of different microalgae species, a screening step is needed for secondary metabolites and bioactive compounds that could be produced by a species and affect the growth of other members of the consortium (Smith et al. 2010; Patel et al. 2017, pp.109-126). Another point to consider in the synthetic algal consortium is that different species should be mixed together in a ratio in which it can develop a successful synthetic consortium (Patel et al. 2017, pp.109-126).

3.2 Aim and objectives

Bioprospecting for algal strains from WW for WW treatment and biomass utilisation has been done before in many countries including the UK (Osundeko, Davies and Pittman, 2013), the USA (Zhou et al. 2011), India (Renuka et al. 2013), Germany (Su, Mennerich and Urban, 2012) and Australia (Frampton et al. 2013). To increase the probability of a successful WW treatment using microalgae, this project similarly aimed to identify green microalgal strains already adapted to the local WW and environment of both the UK and Jordan. We anticipated, that in addition to high growth rates and associated high nutrient assimilation rates, such strains would possess certain other useful characteristics, in particular tolerance to abiotic factors such as light intensity and availability, temperature and biotic factors such as predators and competitors. Furthermore, before starting bioprospecting we made two further decisions 1) that the algae would be grown in an open pond system and not a closed photobioreactor, and 2) gravity settling would be used to harvest algal biomass from treated water.

The specific objectives addressed in this Chapter are:

1. Obtain monocultures of microalgae isolates potentially suitable for waste water treatment from a) two the UK waste water treatment Plants (WWTPs) close to Bath, and b) from multiple locations in Jordan including several WWTPs.

2. Attempt to identify isolates to either the genus or species level using morphological methods and DNA barcoding based on the 18S rDNA and ITS1-5.8S-ITS2.

3. To find if the obtained microalgae isolates from the UK and Jordan related to each other.

3.3 Results:

3.3.1 Obtaining microalgae monocultures

Bioprospecting for native microalgae, that are adapted to the local environments in the UK and Jordan was the first step for using the microalgae in WW treatment in open ponds. These indigenous microalgae isolates are expected to tolerate the different WW conditions such as, nutrient concentrations, pH, temperature and predation.

To isolate the indigenous microalgae, different water samples were collected from different locations from the UK and Jordan. During January-February in 2014 samples were collected from the secondary treatment effluent from Somerton and final effluent from Avonmouth WWTPs both operated by Wessex Water, which is the water supply and sewerage utility company serving the South West of England. In April-May 2014 samples were collected from various locations in Jordan (Table 2.7). Samples were cultured in blooming condition in the growth room (section 2.1.3) in a nutrient rich medium (BBM) to encourage the growth of a wide range of algal species. In order to obtain single colonies to facilitate isolation of individual species, cultures were subjected to serial dilution. Generally, the most effective dilutions were in the range 1×10^{4}-10^{6} which gave many single colonies were easily visually verified as containing as single species by light microscopy (Figure 3.3).

A random selection of 45 colonies, that are different in colour shape and size were chosen from the UK and the same number from Jordan. Although variation in colony shape, size and colour were considered in making selections, it was assumed that such differences did not necessarily guarantee different species. A morphological examination was performed for the cells of each colony under the light microscopy to document differences in size, shape, colour, and the presence of distinctive morphological features such as spines or flagella (Figure 3.3). It is worth noting that for the purposes of the application we were interested in obtaining green microalgae since this group of species is documented as growing well in WW stabilization ponds (Palmer, 1974, cited in Palmer, 1977). Green algae generally outcompete slower growing macroalgae and are not affected by a requirement for the silica in the WW as is the case for diatoms. After screening the 45 colonies for the variation in the morphology the number was reduced to 12 and 17 samples from UK and Jordan respectively were chosen to be bar coded to confirm the difference.

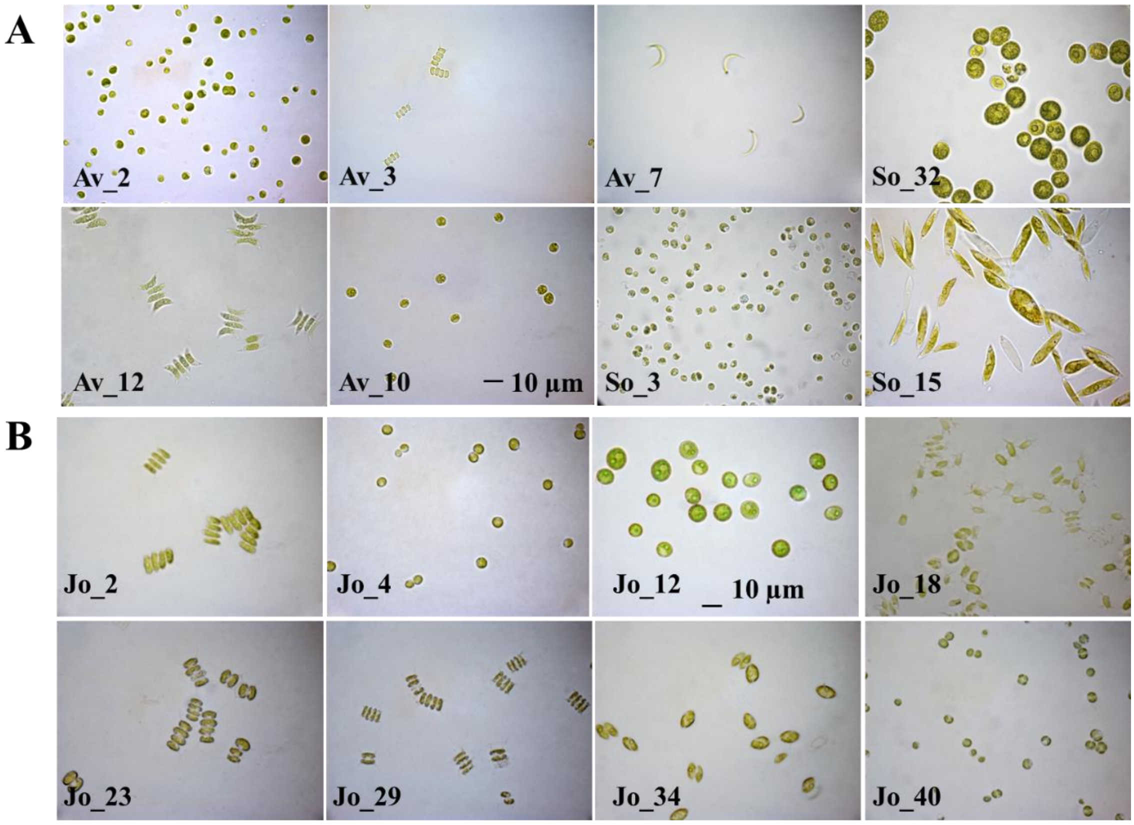

Figure 3.3: Different morphological characters of strains isolated from UK and Jordan. (**A**) Eight microalgae isolates were obtained from Avonmouth (Av_) and Somerton (So_) WWTPs in the UK. (**B**) Eight microalgae isolates were obtained from Jordan, Asamra WWTP (Jo_2 and Jo_4), Al- Karak WWTPs (Jo_12 and Jo_18) Al-Fuheis WWTP (Jo_40), King Abdullah Canal (Jo_23), Fresh water spring from Al-Fuheis (Jo_29 and Jo_34). Microalgae photos are at1000X magnification of the compound light microscope, scale bar = 10 µm.

3.3.2 Amplifying the DNA bar coding markers

Identifying the microalgae isolates is essential to discriminate between the different microalgal species. In addition, it will help in understanding the interaction between microalgae and their environment. Green microalgae are superior for WW treatment, but given difficulties in morphologically identifying microalgae (Darienko et al. 2015), a reliable way is to identity to genus or species level using DNA barcoding. For molecular characterization of microalgae isolates 18S rDNA and ITS1-5.8S-ITS2 where chosen because 18S rDNA is conserved and widely used for algae identification (Pröschold and Leliaert, 2007, pp.123-153; Leliaert et al. 2012) that means there will be more data available in the GenBank than other for genes. ITS1-5.8S-ITS2 is used because it is more variable than18S rDNA and ITS2 has a conserved folding pattern (Buchheim et al. 2011). Targeting these sequences attempts to strike a balance between the need for sufficient sequence conservation, and sufficient sequence polymorphism to enable resolution down to at least the genus, and preferably the species, level.

The average length for the 18S rRNA in eukaryotes is 2kb and the longest 18S rRNA is for the euglenid *Distigma sennii*, comprising >4.5 kb (Torres-Machorro et al. 2010). From all the UK microalgae samples taken forward (Av_2, 3, 7, 10, 12 and So_3,15,32), the amplified 18S rDNA gave the expected band length around 1.7 kb depending on the primers used. There was no variation in the length of the 18S rDNA fragment within the UK isolates. In contrast, such variation was present among the isolates taken forward from Jordan, three isolates had larger fragment ≥2 kb (Figure 3.4 A), Jo_2 (2366 bp), Jo_4 (2346 bp) and Jo_23 (1959 bp). To determine why a subset of Jordan isolates gave longer 18S rRNA gene fragments than the other Jordan isolates, primers were designed to produce full length sequences 18S rRNA sequences. The edited and aligned full length sequences revealed that Jo_4 contains two introns within its 18S rRNA sequence (Figure 3.5): the first is 379 bp in length and located 453 bp from the beginning of the sequence and the second is 436 bp in length and located 1397 bp from the beginning of the sequence. In contrast to Jo_4, Jo_2 and Jo_23 have only one intron which is in the same position as the second intron of Jo_4 (located 1018 bp from the beginning of those sequences). The sequence and length (393 bp) of these Jo_2 and Jo_23 introns are identical sequences, whist the Jo_4 intron is longer (436 bp) and has around 73% identity to the Jo_2 and Jo_23 introns. Several eukaryotic microorganisms in fungi kingdom and the protists contain intron insertions in the nuclear ribosomal RNA (rRNA) genes. Such insertions are called group I introns and interrupt the LSU and SSU and are to act as ribozymes, with several reported to self-splice *in vitro* naked RNA (Einvik, Elde and Johansen, 1998). The presence of group I introns in the microalgae has been reported previously, Table 3.2 provides some examples.

It worth mentioning that Jo_2 isolate has the longest obtained sequences of 18S rRNA (2366 bp) although it has only one intron. This extra sequence which is around 400 bp that were amplified by the primers is located at the end (Appendix 1. A), of which around 285 bp are identical to the beginning of the ITS1-5.8S-ITS2 sequence obtained from J_2 when it was sequenced and aligned as described in the following section.

The second genomic region used in this research for algae identification was the ITS1-5.8S-ITS2 which is known to be more variable than 18S rDNA (Leliaert et al. 2014). The length of ITS1 ranges from 100-400bp, and ITS2 200-500 bp with the longest ITS1 sequence found in *Euglena gracilis* at 1188 bp. This unusually long fragment could be partially explained by the occurrence of two copies of a 37 bp sequence, showing a 70% sequence identity to each other and by another direct repeat of about 100 bp with the two copies being 83% identical to each other in this rDNA (Torres-Machorro et al. 2010). The size of the PCR products varied between different isolates ranges 600 bp to 1500 bp (Figure 3.4 B). The largest PCR product was obtained from So-32 at around 1500 bp. Torres-Machorro et al. (2010) have stated that whilst useful in phylogenetic studies of closely related species, the biological relevance of ITS length variation and the function of internal repeats is not understood.

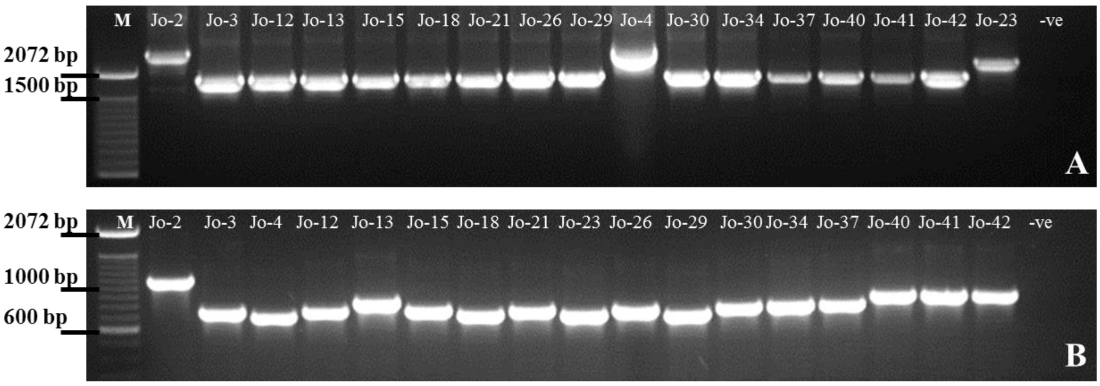

Figure 3.4. Amplified 18SrRNA and ITS1-5.8S-ITS2 sequences from Jordan isolate. **(A)**The partially amplified 18S rDNA from 17 isolates, Jo_2, Jo_4 and Jo_23 have a larger PCR product size than the remaining isolates. **(B)** The amplified ITS1-5.8S-ITS2 region varied in size between the different microalgae isolates. PCR products were analysed on 1% agarose gel in 1X TAE buffer and stained with 0.5 µg/mL ethidium bromide. M is for100 bp DNA ladder (Invitrogen TM life technologies).

Table 3.2. Group I rDNA introns reported in microalgae. modified from (Torres-Machorro et al.2010)

Microalgae	Location	No. of introns	Size (bp)	References
Ankistrodesmus stipitatus	SSU	1	394	D´avila-Aponte et al. 1991
Characium saccatum	SSU	1	477	Wilcox et al. 1992
Chlorella ellipsoidea	SSU LSU	1 1	442 445	Tadanori et al. 1994 a Tadanori et al. 1994 b
Dunaliella parva	SSU	2	381 419	Wilcox et al. 1992
Dunaliella salina	SSU	1	397/8*	Wilcox et al. 1992
Desmodesmus brasiliensis GS2i	SSU-ITS2	3	-	Hoshina, 2014
Desmodesmus opoliensis GS2j	SSU-ITS2	1	-	Hoshina, 2014
Desmodesmus sp. GM4c	SSU-ITS2	2	-	Hoshina, 2014
Desmodesmus bicellularis GM4g	SSU-ITS2	3	-	Hoshina, 2014
Tetranephris brasiliensis GS3f	SSU-ITS2	1	-	Hoshina, 2014
Pectinodesmus sp GB1	SSU-5′LSU	1	-	Hoshina, 2014
Nephrochlamys subsolitaria	SSU-5′LSU	3	-	Hoshina, 2014
Jo_2 (*Desmodesmus sp*.)	SSU	1	393	This study
Jo_4 (*Desmodesmus sp*.)	SSU	2	379 436	This study
Jo_23 (*Desmodesmus sp*.)	SSU	1	393	This study

*Can have two types of intron differing in sequence

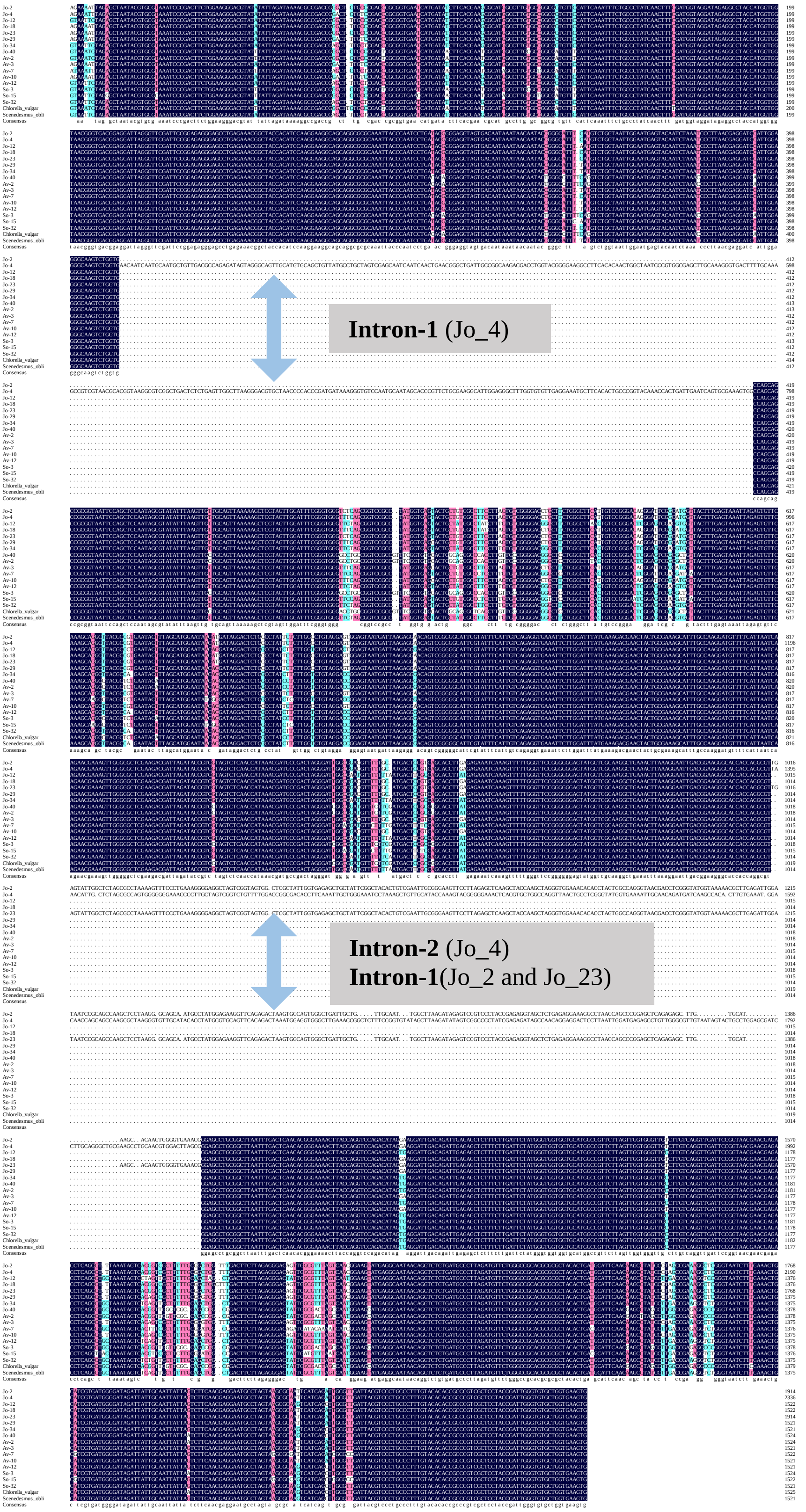

Figure 3.5: Introns in the sequences of 18SrDNA gene of Jo_2, Jo_4, and Jo_23. Multiple alignments for all the UK and Jordan isolates in addition to two references (*Scenedesmus obliquus* and *Chlorella vulgaris*) the arrows indicate the intron position. The multiple alignments of the DNA sequences has been done using the DNAMAN^{TM} Software (Lynnon Biosoft, Quebec, Canada, Version 5. 2. 9).

3.3.3 The microalgae isolates belong to six different genera from Chlorophyta

For the purpose of identifying the obtained microalgae isolates. The amplified PCR products for the both regions (18S rDNA and ITS1-5.8S-ITS2) were purified and sent for sequencing. For each isolate, two samples were sequenced, one with the forward primer and the other with the reverse primer. The resulting sequences were trimmed to remove the bad quality data from the beginning and end of the sequence. The forward and reverse sequences were aligned to each to confirm that the sequence was correct.

Sequences were then subjected to nucleotide BLAST search, and results arranged according to their maximum scores with highest coverage and identity (Table 3.3). nucleotide BLAST takes the query (input DNA sequence), and searches DNA database for identity that ranges from perfect matches to very low similarity. The results come up in the form of the table include scores (Max and Total Score), query coverage %, identity % and the E value (Agostino, 2012). The Max Score shows the highest alignment score obtained for the query sequence among the database sequences (NCBI News, 2006). The Total Score is important, if there are multiple sections of similarity between the query and the hit (identified similar gene sequences) but they are not joined. If the Total Score and the Max Score are equals this means, there is only a single alignment. When the Total Score value is greater than the Max Score there is a multiple alignments like the case of Jo_2 (Agostino, 2012). The query coverage shows the percentage of the query sequence that was involved in the aligned segments. The percentage of the identity, shows to which extent the sequences have the same residues at the same location in the alignment (Fassler and Cooper, 2011). Lastly, the E value (Expect value), provides the number of hits that are expected to be found by chance due to the quality of the alignment besides the size of the database. If the E value number close to or equal zero the hit is significant and not due to chance (Agostino, 2012). For all the isolates that are presented in table 3.3, the E value was zero.

The nucleotide BLAST results were as the following: from the UK isolates, four were identified at the species levels (Av_2, 12 and So_3, 32) and from the Jordan isolates two were confirmed at the species level Jo_34 and Jo_40. The UK isolates (Av_3 and 10) and Jordan isolates (Jo_2, 4, 12, 18, 23 and 29) were confirmed at the genus level. The UK isolates (Av_7and So_15) were not confirmed at the genus level yet (Figure 3.6). The identified species were belonging to three genera *Chlorella*, *Scenedesmus and Scotiellopsis*. Starting with *Chlorella* it includes *Chlorella luteoviridis* (Av_2) and *Chlorella sorokiniana* (So_3 and Jo_40), *Scenedesmus* includes *Scenedesmus obliquus* (Av_12 and Jo_34*)*, and *Scotiellopsis* with *Scotiellopsis reticulata* (So_32).

Unfortunately, the species level for Av_3 and Av_10 from the UK and from Jordan (Jo_2, 4, 12, 18, 23 and 29) was unidentified. The species level was hard to identify for various reasons: the 18S rDNA is very conserved cannot therefore distinguish between closely related species (Krienitz and Bock, 2012) too little polymorphism to allow distinguish closely related species. Although the ITS1-5.8S- ITS2 region is more variable, in some cases it was hard to obtain high identity with a well identified species (i.e. In many cases the sequences were submitted only with the genus level Table 3.3), due to the lack of accurate classification of the submitted samples. A further reason for limited resolving power is the relatively poor representation of algae in GenBank. The available sequences are limited and do not represents all the algal taxa at least for the ITS1-5.8S- ITS2 region. In addition to the above-mentioned points, is that algae taxonomy and classification is changing. You can find the same organism with different species and genera names [e. g *Monoraphidium griffithii* Homotypic Synonym(s) *Closterium griffithii, Ankistrodesmus falcatus* var. *acicularis*]. The results of BLAST searches are summarized in Table 3.3 for the UK isolates and for Jordan isolates.

Two of the UK isolates were not confirmed at the genus level (So_15 and Av_7). Somerton-15 (So-15) showed 100% identity with *Podohedriella falcate* which is synonymous to *Ankistrodesmus falcatus* SAG -202-2, with a Max Score and identity slightly higher than *Monoraphidium neglectum*_SAG 48.87 for the 18S rDNA. The ITS1-5.8S-ITS2 sequences failed to provide a high identity for both genera (Table 3.3). After trimming the sequences for both 18S rDNA and ITS1-5.8S-ITS2 and combining them, the BLAST analysis showed 98% identity with 99% query coverage to *Monoraphidium griffithii* KLL-G017 (homotypic synonyms: *Closterium griffithii* and *Ankistrodesmus falcatus* var. acicularis) with a higher Max Score than *Ankistrodesmus gracilis and Kirchneriella lunaris*. Thus So-15 it may belong to *Monoraphidium* or *Ankistrodesmus.* Since So_15 has 100% identity with 100% query coverage and maximum score for the 18S rDNA sequence with *Ankistrodesmus falcatus* SAG-202-2 more than *Monoraphidium neglectum* SAG 48.87. This favours So_15 belonging to *Ankistrodesmus*.

Avonmouth_7 had 100% identity with the same Max Score with *Monoraphidium contortum and Monoraphidium pusillum* (*Ankistrodesmus braunii* var. *pusillus*) for the 18S rDNA. Similar to So-15 there a high confidence identity was not obtained from the ITS1-5.8S-ITS2 sequences. After trimming and combining the two sequences the highest identity, query coverage and Max Score was for unidentified chlorophyte followed by *Ankistrodesmus gracilis* with higher maximum score and identity than *Monoraphidium griffithii* KLL-G017 (Homotypic synonyms: *Closterium griffithii* and *Ankistrodesmus falcatus* var. *acicularis*). Av_7 may thus belong to *Ankistrodesmus* and *Monoraphidium* genera as found for So-15. Although there was a marked difference in the shape of these two isolates (Figure 3.3 A) they are nevertheless closely related at the molecular level. It may worth noting that the 18SrDNA sequence favours Av_7 belonging to *Monoraphidium* (Table 3.3).

Table 3.3: BLAST results for UK and Jordan isolates

Isolate	Alignments (18SrRNA)	Max/total score	Query	Identity	Alignment (ITS)	Max/total score	Query	Identity	Alignments (18SrRNA and ITS)	Max/total score	Query	Identity
Somerton_3	*Chlorella sorokiniana* FM205834.1	2861*	100%	100%	*Chlorella sorokiniana* KJ676111.1	1304*	100%	100%	*Chlorella sorokiniana* NIES:2173 AB731602.1	2817/3521	100%	100%
	Chlorella sorokiniana X62441.2	2857*	100%	99%	*Chlorella sorokiniana* KJ676109.1	1158*	100%	96%	*Heynigia dictyosphaerioides* GQ487221.1	2796/3311	99%	99%
Somerton_15	*Podohedriella falcate* X91263.1 Homotypic synonym *Ankistrodesmus falcatus* SAG -202-2	2857*	100%	100%	*Scenedesmus sp.* KF471115.1	898*	90%	98%	*Monoraphidium griffithii* KLL-G017 KP726253.1 Homotypic synonyms *Closterium griffithii* *Ankistrodesmus falcatus* var. *acicularis*	3686/3261	99%	98%
	Monoraphidium neglectum SAG 48.87 AJ300526.1	2809*	100%	99%	*Monoraphidium sp.* KF537767.1	819*	90%	95%	*Ankistrodesmus gracilis* AB917098.1	2663/3184	99%	98%
					Ankistrodesmus falcatus UTEX 101	Evalue 2e-162	100%	87%	*Kirchneriella lunaris* strain KLL-G007 KP726238.1	2619/3139	99%	98%
Somerton_32	*Scotiellopsis reticulata* JX513885.1	2942*	100%	100%	*Scenedesmus rubescens* SAG 5.95 HG514422.1	1072*	100%	99%	*Scotiellopsis reticulata* CCALA 474 JX513885.1	2811/3601	100%	100%
	Scenedesmus rubescens KU057946.1	2942*	100%	100%	*Scotiellopsis reticulata* JX513885.1	1072*	100%	99%	*Scenedesmus obliquus* CCAP 276/7 FR865737.1	2795/3477	99%	99%
									Scenedesmus incrassatulus CCAP 276/43 FR865722.1	2795/3466	99%	99%

Isolate	Alignments (18SrRNA)	Max/total score	Query	Identity	Alignment (ITS)	Max/total score	Query	Identity	Alignments (18SrRNA and ITS)	Max/total score	Query	Identity
Avonmouth_2	*Chlorella luteoviridis* CCAP 211/5B FR865678.1	2861*	100%	100%	*Chlorella luteoviridis* CCAP 211/5B FR865678.1	955*	100%	99%	*Chlorella luteoviridis* CCAP 211/5B FR865678.1	2819/3593	100%	100%
	Micractinium sp. CCAP 211/11F FM205877.1	2839*	100%	99%	*Chlorella sp. XFZ-1-1* KF689555.1	931*	100%	99%	*Micractinium pusillum* strain CCAP 248/1 FM205874.1	2802/3357	100%	99%
	Chlorella sorokiniana SAG 211-8k X62441.2	2835*	100%	99%					*Dictyosphaerium sp.* CB 2008/108 GQ507371.1	2791/3248	100%	99%
Avonmouth_3 **Avonmouth_10**	*Desmodesmus sp*. GM4a AB917128.1	2844*	99%	100%	*Desmodesmus sp*. MAT-2008c EU502836.1	1048*	100%	100%	*Desmodesmus sp*. GM4a AB917128.1	2813/3582	100%	100%
	Desmodesmus sp. GB1a AB917097.1	2844*	99%	100%	*Scenedesmus sp.* MC-1 KJ740712.1	987*	100%	98%	*Desmodesmus sp. GB1a* AB917097.1	2813/3577	100%	100%
	Scenedesmus abundans UTEX 343 X73995.1	2844*	99%%	100%	*Desmodesmus sp.* GB1a AB917097.1	981*	100%	98%	*Desmodesmus communis* AICB JQ922412.1	2747/3318	100%	99%
	Desmodesmus abundans SAG 2048 KF673371.1	2828*	99%	99%					*Scenedesmus armatus* var. *subalternans* CCAP 276/4A FR865727.1	2737/3355	100%	99%
	Desmodesmus subspicatus SAG 86.81 KF673378.1	2789*	99%	99%								
	Desmodesmus communis AICB *JQ922412.1*	2778*	100%	99%								

Isolate	Alignments (18SrRNA)	Max/total score	Query	Identity	Alignment (ITS)	Max/total score	Query	Identity	Alignments (18SrRNA and ITS)	Max/total score	Query	Identity
Avonmouth_7	*Monoraphidium contortum* AY846382.1	2857*	100%	100%	Uncultured Chlorophyta HQ191424.1	1044*	91%	99%	Uncultured Chlorophyta HQ191424.1	2811/3595	100%	99%
	Monoraphidium pusillum Homotypic synonym *Ankistrodesmus braunii* var. *pusillus* AY846376.1	2857*	100%	100%	*Monoraphidium sp*. KMMCC 1531 JQ315786.1	1044*	91%	90%	*Ankistrodesmus gracilis* AB917098.1	2739/3281	99%	99%
					Monoraphidium griffithii KLL-G017 KP726255.1	737*	91%	89%	*Monoraphidium griffithii* KLL-G017 KP726253.1 Homotypic synonyms *Closterium griffithii* *Ankistrodesmus falcatus* var. *acicularis*	2628/3215	99%	98%
	Uncultured Chlorophyta HQ191424.1	2852*	100%	99%								
	Monoraphidium sp. SDEC-17 KT180321.1	2780*	100%	99%								
Avonmouth_12	*Tetradesmus obliquus* CCAP 276/3A Homotypic synonym(s) *Scenedesmus obliquus* *Acutodesmus obliquus* KU900221.1	3024*	100%	100%	*Acutodesmus obliquus* CCAP 276/48 JQ082318.1	909*	100%	99%	*Scenedesmus obliquus* CCAP 279/46 FR865738.1	2811/3605	100%	100%
					Scenedesmus acutus AJ249509.1	909*	100%	99%	*Scenedesmus incrassatulus* Bohlin Homotypic synonym *Tetradesmus incrassatulus* CCAP 276/43 FR865722.1	2811/3593	100%	100%
	Scenedesmus basiliensis ACKU 646-06 KF898121.1	3024*	100%	100%	*Scenedesmus dimorphus* UTEX 1237 KP645232.1 Homotypic synonym *Tetradesmus dimorphus*	904*	100%	99%				
	Scenedesmus incrassatulus CCAP 276/43 Homotypic synonym *Tetradesmus incrassatulus* FR865722.1	3024*	100%	100%								

Isolate	Alignments (18SrRNA)	Max/total score	Query	Identity	Alignment (ITS)	Max/total score	Query	Identity	Alignments (18SrRNA and ITS)	Max/total score	Query	Identity
Jordan_2	*Desmodesmus sp.*GTD9C2 JQ315186.1	2994/3328	82%	99%	*Desmodesmus sp.* F30 JQ867365.1	1386*	98%	93%	*Desmodesmus sp.* GM4a AB917128.1	2763/3352	100%	99%
	Desmodesmus sp. F2 JF835992.1	2920/325	80%	99%	*Desmodesmus brasiliensis* GS2i AB917106.1	1221*	100%	90%	*Desmodesmus communis* KLL-G008 KP726233.1	2752/3392	100%	99%
	Pediastrum biradiatum UTEX 37 AY663034.1	2401/3419	100%	91%					*Scenedesmus armatus* var. *subalternans* CCAP 276/4A FR865727.1 Homotypic synonym *Desmodesmus armatus* var. *subalternans*	2771/3448	99%	99%
	Scenedesmus sp. NJ-1 JX286515.1	2386/3439	99%	94%								
	Monoraphidium contortum AS-11 AY846375.1	2215/3174	99%	92%					*Desmodesmus intermedius* CCAP 258/36 FR865701.1 Introns were removed	2761/3366	99%	99%
	Chlamydomonas sp. CCAP 11/141 FR865536.1	2736/3017	99%	91%								
Jordan_4	*Desmodesmus brasiliensis GS2i* AB917106.1	3061*	100%	91%	*Desmodesmus multivariabilis* var. *turskensis* Mary 8/18 T-1W DQ417525.1	1040*	100%	98%	*Desmodesmus sp.* GM4a AB917128.1	2774/3357	100%	99%
	Desmodesmus armatus GM4h AB917135.1	3055*	100%	91%	*Desmodesmus sp.* GTD9C2 JQ315188.1	1031*	100%	98%	*Desmodesmus communis* AICB JQ922412.1	2769/3414	100%	99%
	Desmodesmus bicellularis GM4g AB917134.1	3053*	100%	91%	*Desmodesmus sp.* GM4i AB917136.1	1029*	100%	98%	*Scenedesmus armatus* var. *subalternans* CCAP 276/4A FR865727.1	2771/3464	99%	99%
	Desmodesmus pannonicus GM4n AB917139.1	2126/2860	83%	92%	*Desmodesmus subspicatus* GB6 KX389313.1	963*	100%	96%	*Desmodesmus intermedius* CCAP 258/36 FR865701.1	2750/334	99%	99%
					Scenedesmus armatus var. *subalternans* CCAP 276/4A FR865727.1	891	100%	94%	*Desmodesmus bicellularis* KLL-G009 KP726230.1	2754/3352	99%	99%

Isolate	Alignments (18SrRNA)	Max/total score	Query	Identity	Alignment (ITS)	Max/total score	Query	Identity	Alignments (18SrRNA and ITS)	Max/total score	Query	Identity
Jordan_12	*Pseudospongiococcum protococcoides* CALU - 221 KU057947.1	2863*	100%	100%	*Coelastrella sp.* shy-188 KP702302.1	1168*	100%	100%	*Coelastrella sp.* SAG 2123 JX513883.1	2791/3533	100%	99%
	Coelastrella sp. SAG 2471 KM020087.1	2863*	100%	100%	*Chlamydomonas moewusii* JX290025.1	1157*	100%	99%	*Chlorella emersonii* CCAP 211/15 FR865661.1	2785/3398	100%	99%
	*Scenedesmus sp.*Ki4 AB734096.1	2863*	100%	100%	*Scenedesmus sp.* Ki4 AB762691.1	1151*	100%	99%	*Scenedesmus regularis* FR865732.1 Homotypic synonym *Pectinodesmus regularis* CCAP 276/53	2785/3398	100%	99%
	Asterarcys quadricellulare KT280061.1	2841*	100%	99%	*Haematococcus pluvialis* JX046429.1	1151*	100%	99%	*Scenedesmus pectinatus* FR865730.1 Homotypic synonym *Pectinodesmus pectinatus* CCAP 276/51	2769/3379	100%	99%
	Chlorella emersonii CCAP 211/15 FR865661.1	2835*	100%	99%								
	Scenedesmus vacuolatus Homotypic synonym *Coelastrella vacuolata* X56104.1	2832*	100%	99%								
Jordan_18	*Desmodesmus subspicatus* SAG 86.81 KF673378.1	2900*	100%	100%	*Desmodesmus sp.* IB-01 JQ782747.1	1103*	100%	100%	*Desmodesmus bicellularis* KLL-G009 KP726230.1	2771/3340	100%	99%
	Desmodesmus bicellularis KLL-G009 KP726230.1	2856*	100%	99%	*Desmodesmus sp.* F32 JQ867366.1	1068*	100%	99%	*Desmodesmus sp.* GM4a AB917128.1	2785/3285	100%	99%
	Desmodesmus armatus var. *subalternans* SAG 276-13 KF673362.1	2850*	100%	99%	*Desmodesmus subspicatus* GB6 KX389313.1	917	98%	95%	*Scenedesmus armatus* var. *subalternans* CCAP 276/4A FR865727.1	2765/3409	99%	99%
									Desmodesmus intermedius CCAP 258/34 FR865699.1	2760/3331	99%	99%
									Desmodesmus pannonicus CCAP 258/48 FR865712.1	2752/3283	99%	99%

Isolate	Alignments (18SrRNA)	Max/total score	Query	Identity	Alignment (ITS)	Max/total score	Query	Identity	Alignments (18SrRNA and ITS)	Max/total score	Query	Identity
Jordan_23	*Desmodesmus sp.* GTD9C2 JQ315186.1	2994*	83%	99%	*Desmodesmus sp.* GTD9C2 JQ315188.1	1079*	100%	99%	*Desmodesmus sp.* GM4a AB917128.1	2763/3346	100%	99%
	Monoraphidium minutum KMMCC 1521 JQ315548.1	2686*	100%	91%	*Desmodesmus multivariabilis* var. *turskensis* DQ417525.1	1022*	100%	98%	*Desmodesmus communis* KLL-G008 KP726233.1	2752/3387	100%	99%
	Chlamydomonas sp. CCAP 11/141 FR865536.1	2639*	100%	91%	*Desmodesmus subspicatus* GB6 KX389313.1	929*	99%%	95%	*Scenedesmus armatus* var. *subalternans* CCAP 276/4A FR865727.1	2771/3448	99%	99%
	Scenedesmus sp. II22 FJ946899.1	2266/3038	100%	94%					*Desmodesmus intermedius* CCAP 258/34 FR865699.1	2741/3335	99%	99%
Jordan_29	*Desmodesmus sp. GM4a* AB917128.1	2863*	100%	100%	*Desmodesmus sp. GM4a* AB917128.1	1083*	100%	100%	*Desmodesmus sp. GM4a* AB917128.1	2813/3627	100%	100%
	Scenedesmus abundans UTEX 343 Homotypic synonym *Desmodesmus abundans* X73995.1	2863*	100%	100%	*Scenedesmus sp.* MC-1 KJ740712.1	1083*	100%	100%	*Desmodesmus communis* AICB JQ922412.1	2747/3335	100%	99%
					Chlorococcum sp. UTEX 819 KX147347.1	1048*	100%	99%	*Scenedesmus armatus* var. *subalternans* CCAP 276/4A FR865727.1	2737/3333	100%	99%
	Desmodesmus abundans SAG 2048 KF673371.1	2846*	100%	99%	*Desmodesmus asymmetricus* Tow 9/21 P-12W DQ417549.1	926*	100%	95%	*Desmodesmus intermedius* CCAP 258/36 FR865701.1	2728/3281	99%	99%
	Desmodesmus subspicatus SAG 86.81 KF673378.1	2808*	100%	99%								
	Desmodesmus communis Homotypic synonym *Scenedesmus communis* UTEX 76 X73994.1	2793*	100%	99%								

Isolate	Alignments (18SrRNA)	Max/total score	Query	Identity	Alignment (ITS)	Max/total score	Query	Identity	Alignments (18SrRNA and ITS)	Max/total score	Query	Identity
Jordan_34	*Tetradesmus obliquus* CCAP 276/3A KU900221.1 Homotypic synonyms *Acutodesmus obliquus* *Scenedesmus obliquus*	2902*	100%	100%	*Acutodesmus obliquus* strain UTEX 1450 KP645234.1	1179*	100%	100%	*Acutodesmus obliquus* GB1g AB917101.1	2811/3610	100%	100%
					Scenedesmus dimorphus UTEX 1237 KP645232.1	1179*	100%	100%	*Scenedesmus obliquus* CCAP 276/7 FR865737.1	2811/3616	100%	100%
	Scenedesmus bijugus var. *obtusiusculus* KJ808696.1	2902*	100%	100%	*Acutodesmus bernardii* CCAP 276/38 JQ082329.1	1179*	100%	100%	*Scenedesmus incrassatulus* CCAP 276/43 FR865722.1	2811/3605	100%	100%
	Scenedesmus obliquus CCAP 276/7 FR865737.1	2902*	100%	100%	*Scenedesmus naegelii* UTEX 74 AJ249510.1	1175*	100%	99%	*Scenedesmus obliquus* CCAP 276/52 FR865731.1	2800/3492	100%	99%
	Scenedesmus incrassatulus CCAP 276/43 FR865722.1	2902*	100%	100%	*Acutodesmus obliquus* strain UTEX 393 KP645233.1	1173*	100%	99%				
Jordan_40	*Chlorella sorokiniana* UTEX 2714 LK021940.1	2950*	99%	100%	*Chlorella sorokiniana* UTEX 3010 KY229196.1	1303*	100%	100%	*Chlorella sorokiniana* UTEX 2714 LK021940.1	2817/3627	100%	100%
	Chlorella chlorelloides CB 2008/110 HQ111432.1	2944*	99%	99%	*Chlorella vulgaris* UTEX 2714 KP645229.1	1297*	100%	99%	*Chlorella sp*. IFRPD1018 AB260898.1	2817/3470	100%	100%
									Chlorella sorokiniana SAG 211-8k. FM205834.1	2811/3472	100%	99%
	Chlorella lewinii *CCAP 211/90* FM205861.1	2944*	99%	99%	*Chlorella sp.* ZJU0204	0.0	99%	100%				
					Chlorella sorokiniana UTEX:3011 KY229197.1	983*	100%	92%	*Chlorella chlorelloides* CB 2008/110 HQ111432.1	2811//3368	98%	99%
	Heynigia riparia *CCAP 222/47* GQ487225.1	0.0	99%	99%								
									Chlorella lewinii CCAP 211/90 FM205861.1	2811/3411	98%	99%

- E-values were 0.0; *the total and the maximum scores are the same, if not they are sated as maximum score/ total score; Accession numbers are in blue

3.3.4 Constructing phylogenetic tree for both the UK and Jordan isolates

To clarify the relationship between the isolates obtained from each country. As well to find if there is a phylogenetic relationship between the isolates that are obtained from the UK and the isolates from Jordan. A phylogenetic tree based on the maximum likelihood for the two regions together (18SrDNA and ITS1- 5.8S-ITS2) was constructed for all the isolates from the UK, Jordan and for three reference sequences from the GenBank: *Chlorella vulgaris* SAG 211-11b (X13688.1), *Scenedesmus obliquus* CCAP 276/7 (FR865737.1) and *Chlamydomonas reinhardtii* CCAP 11/32CW15 (FR865576.1) (Figure 3.6). After trimming, removing the introns from (Jo_2, 4, 23) combining the sequences, they were aligned together to construct the tree (Appendix 1. B). The UK isolates were spread across 5 genera*: Chlorella*, *Desmodesmus*, *Scotiellopsis*, *Scenedesmus* (*Acutodesmus*) *and Monoraphidium* or *Ankistrodesmus*. In contrast, the isolates obtained from Jordan were slightly less diverse, belonging to 4 genera, with 5 out of 8 isolates belonging to *Desmodesmus,* whilst the rest were *Chlorella*, *Scenedesmus* (*Acutodesmus*), and *Coelastrella*.

In Table 3.3, Somerton_32 (So_32) gave 100% identity for the 18S rDNA and 99% identity for ITS1-5.8S-ITS2 in both *Scotiellopsis reticulata* and *Scenedesmus rubescens* with the same Max Score. This was unexpected since these species belong to different genera. However, this close relationship has been recorded previously by Kaufnerová and Eliáš (2013) who used the 18S rDNA and ITS2 region and concluded that 'they are closely related and if not conspecific'. A close relationship between the genera *Scotiellopsis* (So_32) and *Coelastrella* (Jo_12) was previously recorded in (Punčochářová and Kalina, 1981; Hegewald and Hanagata, 2000; Kaufnerová and Eliáš, 2013). This lends further support for placing *Scenedesmus*, *Scotiellopsis and Coelastrella* close to each other in the phylogenetic tree (Figure 3.6).The relationships between isolates obtained from Jordan and UK is summarised in Figure 3.6. Many clades have samples from Jordan and the UK, which means that those samples are related to each other. The tree shows that Av_12 and Jo_34 appear closely related; Av_3, Av_10 and Jo_29 are more related to each other than the rest of *Desmodesmus* isolates and this is also the case for So_3 and Jo_40 and So_32 and Jo_12. It worth noting that even where the Jordan and the UK isolates are identified as the same species there are nevertheless differences in the sequence and behaviour.

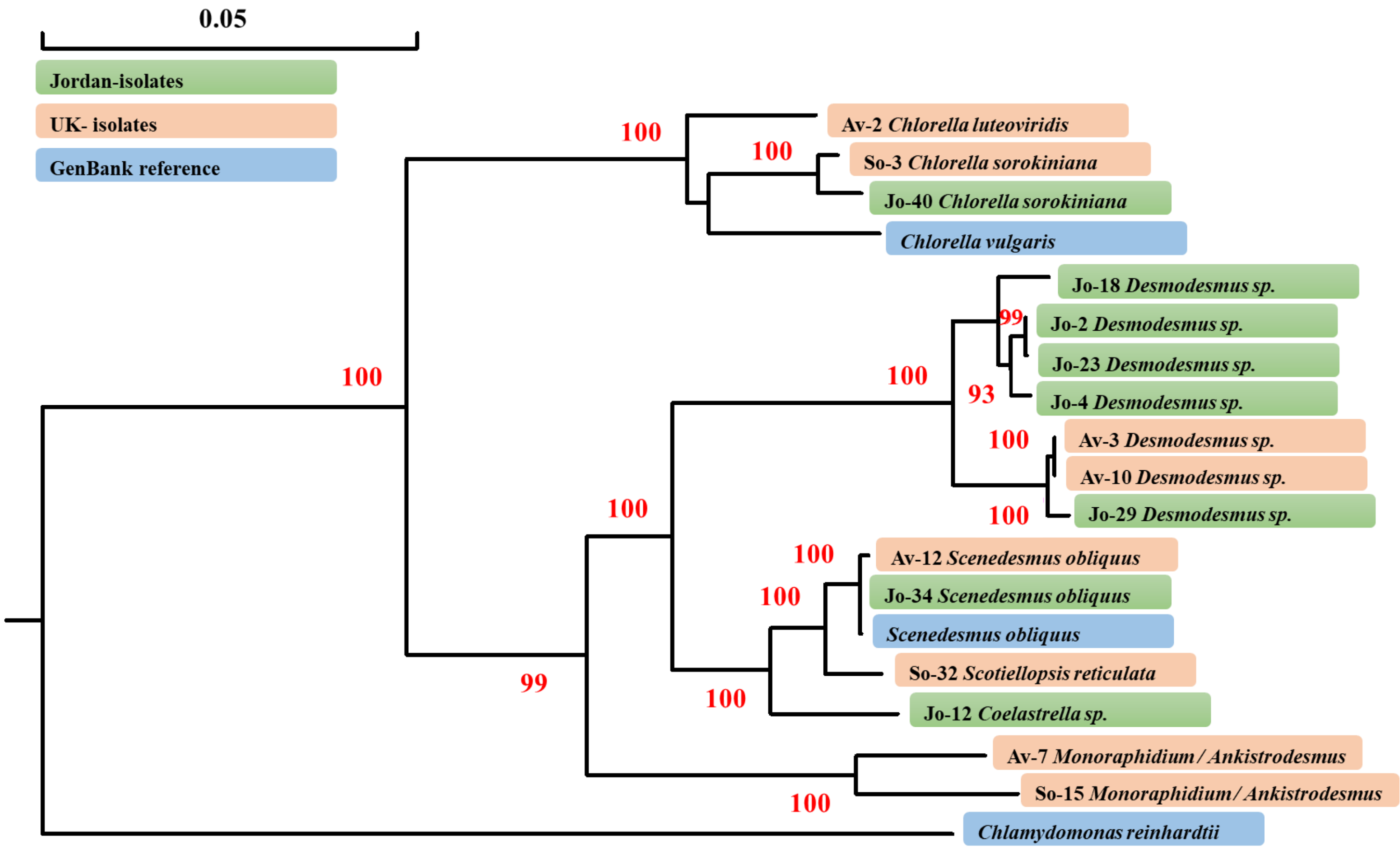

Figure 3.6: Phylogenetic relationship of the UK and Jordan isolates. A phylogenetic analysis of 18S rDNA and the ITS1-5.8S-ITS2 nucleotide sequence was obtained for the 8 UK and 8 Jordan isolates and three references sequences obtained from GenBank (*Chlorella vulgaris* SAG 211-11b, *Scenedesmus obliquus* CCAP 276/7 and *Chlamydomonas reinhardtii* CCAP 11/32CW15). The tree was generated using maximum likelihood analysis of aligned sequences at 1000000 bootstraps by the DNAMAN^{TM} Software. Bootstrap values are indicated at the tree nodes of branches. The branch length scale bar indicates evolutionary distance.

3.4 Discussion

This part of the project aimed to bioprospect for indigenous green microalgae in WWTPs the UK and Jordan to be applied for WW treatment in HRAPs. The classical dilution method for isolating microalgae was easy to apply and effective to obtain a single colony. the obtained colonies where subcultured 2-3 times to ensure the purity of the isolates. Eight isolates from the UK and another eight from Jordan were identified, these isolated were characterized using the 18S rDNA and ITS1-5.8S-ITS2 sequences. A phylogenetic tree was constructed to illustrate the relationship between these isolates.

The obtained UK isolates were morphologically more variable than Jordan isolates in the shape colour and size. The shape of algal cells ranged from spherical (Av_2), spindle Av_12, and needles like crescent shape Av_7, furthermore the cells could be found solitary like So_3 to colonial in a form such as Av_3. In addition to the shape, there was variation in the size (So_3 and So_32) and the colour from light green in Av_7 to dark green in So_32 (Figure 3.3 A). The Jordan isolates were distinguished by the presence of the spines on the cells such as Jo_18, 29 and 23, the colonial formation was more abundant in the isolates of Jordan (Jo_2, 18, 23, 29 and 34) these could be used as defence mechanism. The cylindrical cell shape was the most common shape (Figure 3.3 B).

It is difficult to classify the green microalgae depending on their morphology alone, because some microalgae such as the coccoid do not have distinguished morphological features (Krienitz and Bock, 2012). In addition the morphology of some microalgae varied with the different environmental conditions (Luo et al. 2006). The molecular identification of the isolates based on 18S rDNA and ITS1-5.8S-ITS2 sequences was chosen because it is more reliable and easy method for identifying microalgae at the genus and species levels (Krienitz and Bock, 2012).

The microalgae isolates from the UK and Jordan belong to at least six different genera (*Chlorella, Desmodesmus, Scenedesmus, Scotiellopsis, Coelastrella. and Monoraphidium* or *Ankistrodesmus*). Three genera were common between the UK and Jordan isolates which are *Chlorella, Desmodesmus*, and *Scenedesmus*. The UK isolates were more diverse with at least two genera with a total number of genera was 5 (*Scotiellopsis, and Monoraphidium* or *Ankistrodesmus*). However, the isolates obtained from Jordan were less diverse, belonging to 4 genera in addition to the previously mentioned the genus *Coelastrella* was identified.

3.4.1 *Desmodesmus, Scenedesmus* and *Chlorella* are common genera between the UK and Jordan isolates

In this research, *Scenedesmus* (*Acutodesmus*) and *Desmodesmus* were common in the pool of isolates resulting from bioprospecting at WWTP, with genera/species representations of 25% in the UK and 60% in Jordan. *Scenedesmus and Scenedesmus-like* species were re-classified based on ITS2 sequence analysis into two genera *Scenedesmus* and *Desmodesmus* (An, Friedl and Hegewald, 1999). Subsequently, many species have been transferred to *Desmodesmus* such as *Scenedesmus abundance* became *Desmodesmus abundance* along with another 31 species and 22 varieties (Hegewald, 2000). *Acutodesmus* was kept as subgenera in *Scenedesmus* by (An, Friedl and Hegewald, 1999). However, whilst *Acutodesmus* has been suggested as a new genus by Tsarenko and Petlevanny (2001), Hegewald and colleagues using analysis of ITS2 region support keeping the *Acutodesmus* subgenus under *Scenedesmus* (Hegewald et al. 2010; Kaufnerová and Eliáš, 2013). Some of the *Scenedesmu*s species have been renamed *Acutodesmus,* such as *Scenedesmus obliquus,* the reference species used in the present study, and Av_12 and Jo_34 would become *Acutodesmus obliquus* (John, Whitton and Brook, 2002, pp. 384-385). However, the present study will keep the *Scenedesmus obliquus* name for the reference and the Av_12 and Jo_34 isolates. Table 3.3 shows the genus *Tetradesmus* for theses isolates since this genus was merged in *Acutodesmus* by Tsarenko and Petlevanny (2001).

Algae naturally grow well in WW playing an important role in wastewater treatment in lagoons where they help in the conversion of sewage to stable secondary effluent (Palmer, 1977). Algae release oxygen into the water that is necessary for aerobic decomposition of the organic matter by bacteria. A study published by Palmer in 1974 (cited in Palmer, 1977) recorded 125 algal genera in the sewage pond from 74 ponds in 18 states in the United States over a six years period. Around 50% of the recorded algae were a green alga, with pigmented flagellate species the next most prevalent at 25%, followed by the blue-green algae at 15% and the diatoms at 10%. In terms of genera in descending order of prevalence the most common were *Chlorella*, *Ankistrodesmus*, *Scenedesmus, Euglena*, *Chlamydomonas*, *Oscillatoria*, *Micractinium*, *Colenkinia*, *Anacystis*, and *Oocystis*'

(Palmer, 1974). *Chlamydomonas* was the only genus abundant all over the country, and *Chlorella* was rare in the south-eastern states. Some algae have been recorded that are uncommon for sewage ponds such as *Enteromorpha*, *Zygnema*, *Aphanizomenon*, *Pediastrum*, *Dinobryon* and others (Palmer, 1974).

A study that monitored algae abundance in sewage ponds in California (Lancater) over 6 years found that the most consistently abundant algae every month in every year was *Scenedesmus* (Plamer, 1969). *Chlorella* has been reported in Africa to dominate the first stage of treatment (Clausen, 1959).

In the present study, 25% of UK isolates were *Chlorella*. As mentioned above *Chlorella* is one of the genera that commonly found in the WW. In a 2-year seasonal variation study of phytoplankton and zooplankton in high rate algal ponds for domestic WW treatment in the Mediterranean region of southern France (Canovas et al. 1996). The phytoplankton was restricted to 3-12 taxa dominated by *Chlorella* and *Scenedesmus,* with the narrow species abundance attributed to the hypereutrophic conditions. *Scenedesmus* dominated in the spring whilst *Chlorella* dominated in the winter and autumn due to its higher growth rate in low irradiance and when predation was limited (Canovas et al. 1996). The sample collection was performed in January (winter) in the UK and in the spring in Jordan. This may explain the greater prevalence of *Desmodesmus* in the Jordan isolates compared to the UK.

Scenedesmaceae is considered to be the largest coccoid green microalgae group in the freshwater ecosystem (Krienitz and Bock, 2012). This family has around 29 genera including *Scenedesmus* (*Acutodesmus*), *Desmodesmus, Asterarcys* and *Coelastrella* (Hegewald et al. 2010). They have great morphological variability which has been attributed to their mode of reproduction, the non-sexual reproduction by the production of autospores leads to maintain the mutations, that happens and do not affect the growth and competition of other algae (Hegewald, 1997; Krienitz and Bock, 2012). *Scenedesmus* species are the main constituent of the green algal biomass in phytoplankton and geographically widely distributed in fresh and brackish water bodies and especially in nutrient rich environments (An, Friedl and Hegewald, 1999).

3.4.2 The 18S rDNA ITS1-5.8S-ITS2 sequences as markers

One of the most commonly used phylogenetic markers is the 18S rDNA gene -which was used in this study- is a universal gene (Chapman et al. 1998, pp. 508-540; Huss et al.1999; cited in Krienitz and Bock, 2012). The 18S rDNA has conserved sequences that are good for, designing universal primers, and aligning the sequences of not closely related taxa (Long and David, 1980; Sogin et al. 1986 cited in Krienitz and Bock, 2012). Furthermore, it is could be amplified easily by PCR since it presents in multiple copies in the genome. Thus, the 18S rDNA is valuable to identify the classes and orders of the algae (Friedl, 1995; Krienitz et al. 2003, 2011a). However, the 18S rDNA is not useful to distinguish between closely related coccoid green algal species (Krienitz and Bock, 2012).

Barcoding suggests the same species for the UK isolates Av_3 and Av_10, but these isolates are morphologically different. Av_3 and Av_10 have 100% identity with each other for both the 18S rDNA and ITS1-5.8S-ITS2 sequences, they were identified as *Desmodesmus sp*. Table 3.3. They have been treated as two potentially different species due to differences in cell morphology (Figure 3.3 A). Cells of Av_3 are more elongated and frequently present in a colonial form with usually 4 cells arranged end-to-end in a linear array with the two outer most cells having two spines. In contrast, Av_10 cells were more ellipsoidal and were not observed in the colonial form or with spines. However, despite these striking morphological differences the 18S rDNA and ITS1-5.8S-ITS2 sequences had 100% identity for both regions. Av_3 and Av_10 were treated as two isolates of the same species with the intention of determining whether differences in the growth and behaviour in WW could explain the difference in the shape. However, despite reports that the variation of the shape for this genus is widely affected by the environmental conditions (John, Whitton and Brook, 2002, pp. 384-385), differences were observed i.e. Av_10 never became colonial or developed spines. In contrast culturing Av_3 stocks for maintenance in BBM did result in a proportion of single cells without spines.

At the genus level, it was hard to discriminate between Av_7 and So_15. Although they are different in morphology (Figure 3.3 A). They were identified as members of the genera *Ankistrodesmus* and *Monoraphidium*. This close relationship between *Ankistrodesmus* and *Monoraphidium* and other genera of the same family Selenastraceae has been noted previously (Krienitz et al. 2001). Krienitz and his colleagues in 2001 investigated whether, the classification of the genera in Selenastraceae family made previously according to the classical morphological and ultrastructure methods was supported by the results of the analyses of 18S rDNA gene. They found that species in different genera like *Ankistrodesmus* and *Monoraphidium* were polyphyletic (present in in different clades) and not monophyletic, this means that they are not distinguishable as separate genera (Krienitz et al. 2001).

Another phylogenetic marker receiving attention is internal transcribed spacer 2 (ITS2). Although the primary sequence of this region is highly divergent enabling discrimination differentiation between two related taxa at the species and the genus level the ITS2 RNA transcript has a very conserved secondary structure motive among all eukaryotes (Mai and Coleman, 1997; Coleman, 2003, Schultz et al. 2005). It has a specific folding pattern allows the alignment of closely related sequences. This pattern has 4 helices, the most conserved and the longest helix is helix III (Coleman, 2007). The nuclear ITS2 rRNA sequence- structure has been used to construct the green algal tree of life, Figure 3.7 shows the phylogenetic tree for the of phylum Chlorophyta (Buchheim et al. 2011). That study showed that ITS2 is able to resolve the main green algal lineages establishing ITS2 as a potential powerful DNA barcode. It could help solve complicated issues in microbial ecology and diversity by aiding the analysis and evaluating the structure of the community and the issue of algal geographical distribution (Buchheim et al. 2011).

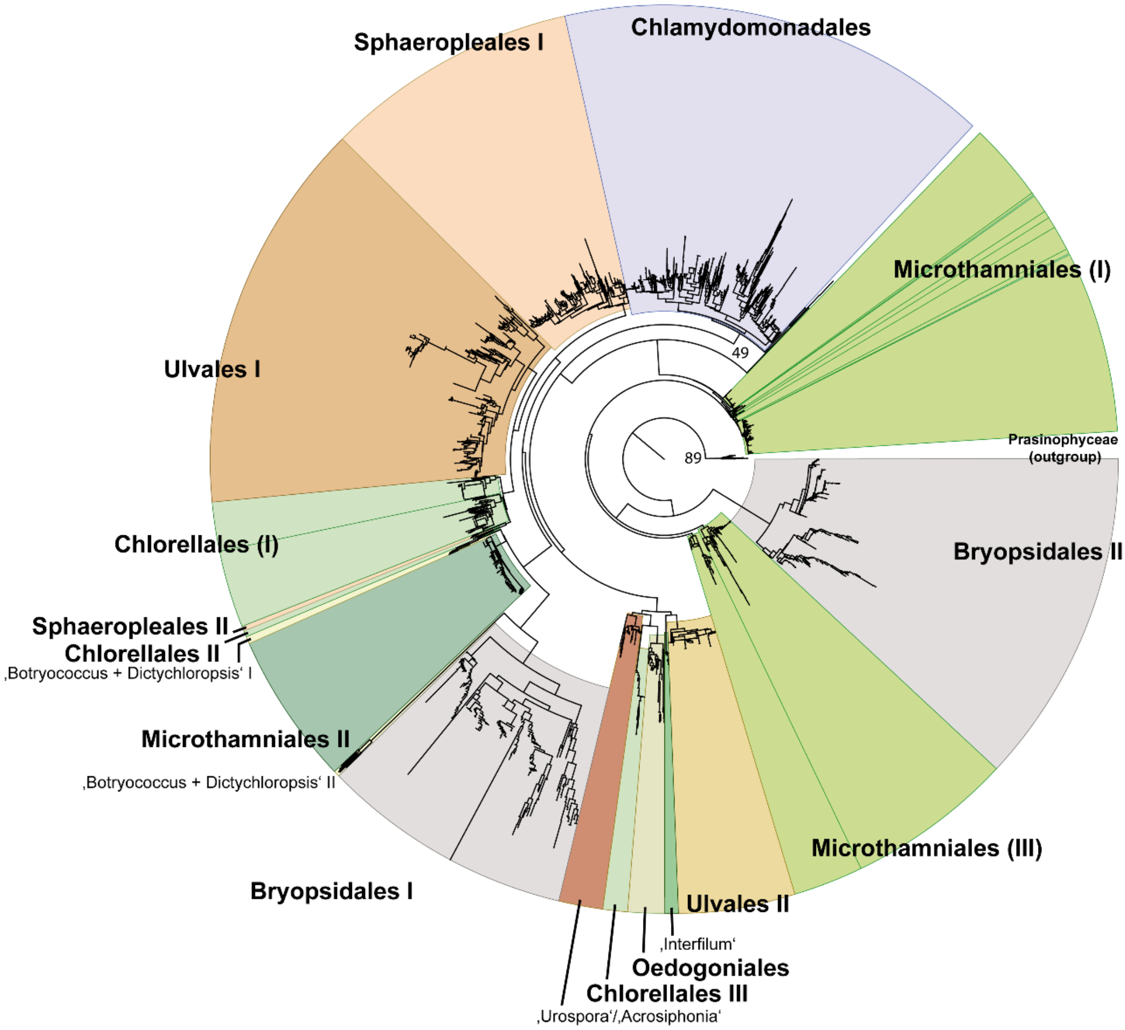

Figure 3.7: Profile Neighbor-Joining (PNJ) tree for sequence-structure data for nuclear ITS-2 rRNA gene of phylum Chlorophyta. Multiple sequence alignment for ITS2 sequences with available secondary structures was performed using 4SALE v1.5 (Seibel et al., 2006; Seibel et al., 2008). The phylogenetic relationships were rebuilt by PNJ tree, based on primary and secondary structure information of ITS2, General Time Reversible (GTR) substitution model, in ProfDistS v0.9.8 (Friedrich et al., 2005; Rahmannet al., 2006; Wolf et al., 2008) differential colour coding and labelling for the major group (Buchheim *et al.*, 2011).

3.5 Conclusion and future work

To achieve a sustainable WW tratment using the microalgae, this reaearch started with exploring the natural diversity of the microalgae to select indigenous microalgae that can be applied for WW treatment and biomass production in open ponds. Species selection is a major step in planning for a successful WW treatment system. In summary, samples were collected from the WWTPs and local environments from the UK and Jordan. Eight indigenous monoculture isolates were obtained from the UK and another eight samples from Jordan. Six genera were identified among the UK and Jordan Isolates (*Chlorella, Desmodesmus*, *Scenedesmus*, *Scotiellopsis, Coelastrella and Monoraphidium* or *Ankistrodesmus*). These genera have been reported before to be present in the WW. There were three common genera *Scenedesmus*, *Desmodsmus*, and *Chlorella*. The majorty of the isolates belonged to *Desmodsmus* and *Chlorella*. Six isolates from the UK and Jordan were identified at the species level, there were difficulties in determining the species and even the genus level for the isolates using only 18S rDNA and the ITS1-5.8-ITS2 sequences. More accurate identification of the isolates would require application of the polyphasic approach integrating morphology, ultrastructure, ecophysiology and the molecuar phylogeny technique.

A general DNA barcoding marker for protists is not yet proposed due to the high diversity among the different potential barcoding genes. One way round this would be to use different barcoding genes for different groups (Darienko et al. 2015). Regarding the SSU rDNA, two variable regions called V4 (Zimmermann, Jahn and Gemeinholzer, 2011) and V9 (Amaral-Zettler et al. 2009) have been recommended. ITS2 is becoming more widely used for identification of the microalgae at the species level. However, it has some limitations regarding the alignment of the sequences and prediction of the secondary structure. The secondary structure of the ITS2 could help in predicting the mating capabilities (Coleman, 2009). The species and the genus could be assigned for microalgae using the presence of compensatory base changes (CBCs) in the secondary structure of the ITS2 sequence (Coleman, 2000). The usage of ITS2 as barcode marker among protists must be validated (Darienko et al. 2015).

Chapter 4

Assessment of microalgae performance in nutrient removal from wastewater and biomass production

4.1 Introduction

4.1.1 Assessment of microalgal performance for wastewater treatment

A bioprospecting process has been performed for microalgae that are native to the WW and local environments in the UK and Jordan for the purpose of WW treatment and biomass production. The bioprospecting process gave eight microalgae isolates from the UK and another eight from Jordan. To apply these microalgae for WW treatment, after identification of the microalgae isolates, the next step is doing a screening process to assess their performance in WW treatment at the small scale, in batch cultures using different criteria. The first criterion is their growth in the WW. The microalgae are preferred to grow fast (has high growth rate) in the WW (Park, Craggs and Shilton, 2011b), this will help the microalga to dominate the WW (Griffiths et al. 2016, pp.269-300) and consume the nutrients for growth. This high growth rate will in turn be reflected in the production of biomass (Park, Craggs and Shilton, 2011b).

The second criterion is the efficiency of removing nutrients (nitrogen and phosphate). Efficient nutrient removal rapid uptake of nutrients from WW, thus reducing WW treatment time and area. Efficient nutrient removal allows microalgae to compete with other microorganisms growing in the WW (Harrison, Parslow and Conway, 1989). There is much ongoing research using microalgae to remove nutrients and produce biomass from WW. Microalgae can be efficient in removing phosphorus, nitrogen, and toxic metals from a wide variety of WW streams (Table 4.1) such as municipal WW (Li et al. 2012; McGinn et al. 2012; Whitton, Ometto and Pidou, 2015). Microalgae could be cultivated in primary or secondary effluent treatment effluent (Table 4.1), agricultural WW piggery (Zhu et al. 2013; Zhu, 2014), and industrial WW such as carpet mill WW (Chinnasamy et al. 2010) and dairy WW (Mulbry et al. 2008; Woertz et al. 2009). Microalgae can be used for heavy metal remediation from WW such as lead and Zinc (Kumar and Goyal, 2010) and organic pollutants from WW (De-Bashan and Bashan, 2010). Microalgae are studied from WW treatment as a single species (Table 4.1) and a consortium of microalgae (Chinnasamy et al. 2010; Samorì et al. 2013)

The third criterion studied is the settleability, determined by observing which isolate(s) settle faster than others by gravity, this is an important advantage to enhance the harvesting process in large scale applications and reduce the cost of harvesting (Park, Craggs and Shilton, 2011b). Harvesting microalgae from large scale WW treatment systems is a major challenge that needs to be considered to achieve an economical WW treatment process and an economical integrated WW treatment process and biofuel production system (Pittman, Dean and Osundek, 2011; Park, Craggs and Shlton, 2013b). Microalgae cells have a negatively charged surface that helps them to form a stable suspension in the water. Furthermore, algae cells have similar density to water, which makes settling of the microalgae from the WW difficult (Craggs et al. 2011). The US Department of Energy (DOE) in 2016, reported that a cost-effective harvesting method is one of the technical challenges and barriers for economical biofuel production from the biomass of microalgae. Mechanical and chemical methods for harvesting like centrifugation, dissolved air floatation (DAF), filtration and chemical flocculation are considered to have high energy demands and are too costly (Benemann and Oswald, 1996; Manheim and Nelson, 2013).

Settling by gravity is a simple and low cost way to harvest microalgae with low energy demands. Above all it does not require skilled operators and it is one of the simplest forms of solid-liquid separation. The major advantages of sedimentation processes are low power consumption, low design cost and low requirement for skilled operators (Craggs, Lundquist and Benemann, 2013, pp153-163). However, there is some disadvantage for this method of harvesting such as requiring a large foot print, a slow sedimentation rate, deterioration of biomass and low solid concentration obtained (Barros et al. 2015). There are many factors that affect the settling of microalgae including size, morphology, motility of microalgal cells, density of the culture and surface charge of the algal cell. In addition to the previous points it is affected by water turbulence and exudate type, composition and concentration have a significant effect on microalgae settling (Henderson, Parsons and Jefferson, 2008; Craggs, Lundquist and Benemann, 2013, pp.153-163). It may worth mentioning that some of the above factors vary between different growth stages in the same species, as well between different species, this may explain the different settling behaviour of microalgae in the same species.

Table 4.1: Nitrogen and phosphorus removal by various genera of green microalgae in batch culture of different WW streams.

Genus and Species	WW stream	Culture type	Tot. N initial concentration (mg/L)	%Tot. N removal efficiency	Tot. P initial concentration (mg/L)	%Tot. P removal efficiency	Reference
Chlorella sp.	Dairy digested manure, diluted	Batch 7 days	100–240	76–83	15–30	63–75	(Wang et al. 2010)
Chlorella pyrenoidosa	IndustrialWW	Fed-batch 5 days	190	89	45.6	70	(Hongyang et al. 2011)
Scenedesmus dimorphus	Industrial, secondary effluent	Batch 9 days	18_a	95_a	56	55	(Gonzalez, Cañizares and Baena, 1997)
Mucidosphaerium pulchellum	Municipal, Primary effluent	Batch 4 days	40_a	100	4.7	100	(Mehrabadi, Farid and Craggs, 2017)
Desmodesmus sp	Municipal, Primary effluent	Batch 4 days	40_a	96_a	4.7	85	(Mehrabadi, Farid and Craggs, 2017)
Chlorella luteoviridis	Municipal, secondary effluent	Batch 5 days	-	80_a	-	80_b	(Osundeko et al. 2013)
Monoraphidium spp. SDEC-17	Industrial WW raw	Batch 16 days	219	56	12	99	(Jiang *et. al.*, 2016)
Chlorella vulgaris,	Municipal primary effluent	Batch 10 days	48.4	90_a	4.29	80_b	(Lau, Tam and, Wong, 1995)
Chlorella zofingiensi	Agriculture WW raw	Batch 10 days	148	78.72	156_b	85_b	(Zhu et al. 2013)

a: nitrogen was measured as ammonium ion (NH_4^+-N)
b: phosphorus was measured as phosphate ion (PO_4^{3-}-P)
% removal range, is due to a range of dilutions of WW (Wang et al. 2010)

4.1.2 Wastewater differs in nutrient composition

Nutrients composition of WW varies with source, affecting the growth of microalgae in and the composition of the biomass (Cai, Park and Li, 2013). The nutrient composition of municipal WW is different from that of the industrial and agricultural WW. For example: municipal WW compared to agricultural (animal) WW has less nitrogen and phosphate, but municipal WW has more nitrogen and phosphate than industrial WW (Cai, Park and Li, 2013). The influent of municipal WW could have a considerable amount of heavy metals such as lead, zinc, and copper, but the industrial WW contains more heavy metal pollutants, so the selection of microalgae that are tolerant to that heavy metal (s) and able to remove it efficiently from the industrial WW is crucial (Ahluwalia and Goyal, 2007; Cai, Park and Li, 2013).

4.1.3 Nitrogen and phosphorus can be utilised by algae

Nitrogen is an important nutrient for the growth of all organisms. Nitrogen is a constituent in many biological substances such as proteins, chlorophylls, energy transfer molecules (ADP, ATP), and genetic materials (Barsanti and Gualtieri, 2006). Algae are able to use inorganic and organic nitrogen. As inorganic nitrogen sources, algae could use ammonium (NH_4^+), ammonia (NH_3), nitrate (NO_3^-), nitrite (NO_2^-), or nitric acid (HNO_3; Cai, Park and Li, 2013). Some cyanobacteria are capable of using N_2 gas to produce ammoniacal nitrogen (NH_4/NH_3) in a process of diazotrophy (Zehr, 2011; Raven and Giordano, 2016, pp.143-154). Algae can also use dissolved organic nitrogen sources such as amino acids (Hellebust, 1978), glycine betaine (Keller et al. 1999), urea (Williams-II and Hodson, 1977), purines (Neilsont and Lewint, 1974), pyrimidines and peptides (Berg, Repeta and Laroche, 2002)

Another essential element for the growth of all organisms is phosphorus. It is a component of genetic material, energy transfer molecules (ADP, ATP), and membranes (Raven and Giordano, 2016, pp.143-154). As is the case with nitrogen, microalgae can utilise inorganic or organic phosphorus. The most common form of inorganic phosphorus that microalgae can utilise is phosphate ion species preferably in the forms $H_2PO_4^-$ and HPO_4^{2-} (Cai, Park and Li, 2013). Studies have reported that pyrophosphate ($P_2O_7^{4-}$) can support the growth of prymnesiophytes such as *Prymnesium parvurn* (Ricketts, 1965; Cembella, Antia and Harrison, 1982). Furthermore, short polyphosphate (tripolyphosphate) can be utilised by two genera of cyanobacteria: *Synechococcus* and *Prochlorococcus* (Moore et al. 2005), and the diatoms of the genus *Thalassiosira* are able to utilise polyphosphates with different chain lengths. Polyphosphates are chains consisting of between three to thousands of orthophosphate groups combined together by phosphoanhydride (P-O-P) bonds (Dyhrman, 2016, pp.155-183; Diaz et al. 2016). Lastly, the phosphite ion (PO_3^{3-}) can be use as the only phosphorus source by the cyanobacteria *Prochlorococcus* strains (Martínez et al. 2011; Feingersch et al. 2012). However, there is no evidence for eukaryotic algae using phosphite (Dyhrman, 2016, pp.155-183).

Algae can utilise dissolved organic phosphorus such as phosphonates that have the C-P bond, and phosphoesters that have the C-O-P ester bond (Lin, Litaker and Sunda, 2016). Dissolved organic phosphates dominate the dissolved inorganic phosphate in many aquatic systems, and the phosphoester dominates the dissolved organic phosphates (Jakuba et al. 2008; Young and Ingall, 2010). As a consequence, the presence of phosphoesterases (e.g alkaline phosphatase, phosphodiesterase, 5′ nucleotidase) play a significant role in fulfilling algal phosphorus demand in many aquatic systems. In addition to that, phosphoesterases are important in phosphorus cycling and recycling at the cellular level (Dyhrman, 2016, pp.155-183). Scanlan et al. (2009) and Martinez et al. (2010) demonstrated that many cyanobacteria can metabolise phosphonates using diverse enzyme systems that are substrate specific, such as phosphonoacetaldehyde hydrolase and wide range specificity C-P lyase enzyme (White and Metcalf, 2007; Villarreal-Chiu et al. 2012; McGrath, Chin and Quinn, 2013).

4.2 Aim and objectives

To use microalgae for nutrient removal and biomass production as a by-product from the secondary effluent of municipal WW in a large scale in the UK and Jordan, it is essential to choose microalgae candidates with favourable criteria for a successful WW treatment system. The aim of this chapter is to assess the growth, nutrient removal efficiency and settleability of the obtained indigenous microalgae isolates from the UK and Jordan when they are grown in secondary effluent WW to come up with some promising strains. To achieve this, microalgae isolates (from the UK and Jordan) were grown in real WW with a reference strain *S. obliquus* CCAP 267/7, obtained from the culture collection. The following specific objectives to be addressed:

1- Assess the growth rate of the microalgae isolates. High growth rate indicates that the microalgae isolate is adapted to the WW and the environmental conditions. Since it grows faster than another microalgae species, it can dominate the WW in which they could be applied. In addition to the previous reasons the faster the microalgae could duplicate their biomass, the more biomass would be obtained in shorter time.
2- Evaluate their efficiency in removing problematic nutrients such as the phosphate and nitrogen from the WW. The microalgae isolate should remove nutrients from the WW quickly so it could reduce the treatment time and compete with other microorganisms for nutrient resources.
3- Assess the settleability of the microalgae isolates under gravity. One of the challenges for applying the microalgae for WW treatment and biofuel production is the cost of harvesting microalgae. Settling by the gravity is a cheaper way of harvesting.
4- Rank the microalgae isolates according to their performance in the above-mentioned criteria (growth, nutrients removal, settleability). To come up with good microalgae candidates for upscaling to treat WW and for biomass production.

4.3 Results

4.3.1 Microalgae isolates growth in wastewater

To achieve a successful open pond WW treatment, fast growing microalgae are required for high productivity in removing nutrients and biomass production. In addition, fast growing microalgae outcompete other microalgae and dominate the pond (Kagami and Urabe, 2001; Borowitzka, 2013, pp.77-89). This section aims to evaluate the specific growth rate of microalgae isolates from the UK and Jordan in a real municipal WW.

So_3 and Av_2 are the fastest growing microalgae among the UK isolates in Somerton WW

To assess the growth of the UK microalgae isolates, the microalgae isolates were grown in batch cultures as triplicates in 400 mL of secondary effluent of Somerton WW, in the growth room optimum growth conditions, supplied with CO_2 and with mixing (section 2.1.3 and 2.2.14). The growth of the microalgae was monitored by daily measurement of the dry weight, cell count and the OD 750 nm. The growth curve for the isolate in WW was built based on the daily measurement of the dry weight (Figure 4.1 A). By the end of the experiment, the maximum dry weight (biomass) obtained was for Av_3, followed by Av_12 (1465 mg/L and 1395 mg/L respectively) as presented Figure 4.1 B. All cultures were seeded with ~1×10^6 cells/mL. The highest cell number by the end of the experiment it was 66×10^6 cells/mL for Av_7, followed by Av_2 (Figure 4.1 C).

How fast the algae grow in WW is an indication for how well it is adapted to that environment. Thus, fast growing algae could dominate their environment (Griffiths et al. 2016, pp.269-300; Prabhakaran et al. 2017, pp. 105-129). The specific growth rate (μ) for the microalgae isolates during the exponential phase has been calculated depending on the change in the DW (section 2.2.15, Equation 1). The average of the specific growth rate is presented in Table 4.2 A. So_3 had the highest specific growth rate followed by Av_2. Both belong to the *Chlorella* genus, whose members are known for their fast growth rate (Canovas et al. 1996). The specific growth rate data were not normally distributed, so a Kruskal-Wallis test (nonparametric test) was applied to detect any significance differences between the medians of the samples. This was followed by homogeneous subsets analysis based on asymptotic significance at significance level 0.05. The results of statistical analysis (Figure 4.1 D) showed that So_3 and Av_2 have the highest specific growth rate and were in the same subset with the reference *S. obliquus*. This means there is no significant difference in their growth in WW. In the other hand, So_3 and Av_2 are significantly different from So_32 and Av_7, which had the lowest growth rates (Figure 4.1 D). Table 4.2 A presents the growth rate data, the generation time (T_g) for the isolates in days and the biomass produced after 9 days. Regarding estimate biomass productivity by the microalgae isolates, the specific growth rate is a good estimate for how fast the algae can duplicate biomass within a specific time in the log phase. The final biomass produced is different from the growth rate results due to the deference in the initial biomass that was seeded.

Table 4.2: Specific growth rate, generation time and biomass (dry weight) produced after 9 days. (A) UK isolates and reference. **(B)** Jordan isolates and the reference.

A				B			
UK Isolates	**μ/ day**	**T_g day**	**DW_9days (mg/L)**	**Jordan Isolates**	**μ/ day**	**T_g day**	**DW_9days (mg/L)**
Av_2	1.030	0.68	1211.90	**Jo-2**	0.653	1.06	1311.82
Av_3	0.830	0.84	1420.97	**Jo-4**	0.787	0.88	935.00
Av_7	0.713	0.97	475.70	**Jo-12**	0.740	0.94	1234.24
Av_10	0.827	0.84	1196.76	**Jo-18**	0.797	0.87	1075.60
Av_12	0.830	0.85	1343.78	**Jo-23**	0.683	1.01	1193.40
So_3	1.193	0.58	767.67	**Jo-29**	1.217	0.57	1662.35
So_15	0.920	0.77	997.65	**Jo-34**	0.963	0.72	1032.65
So_32	0.650	1.07	970.31	**Jo-40**	1.097	0.63	694.00
S. obliquus	0.847	0.82	1166.89	***S. obliquus***	0.793	0.87	934.84

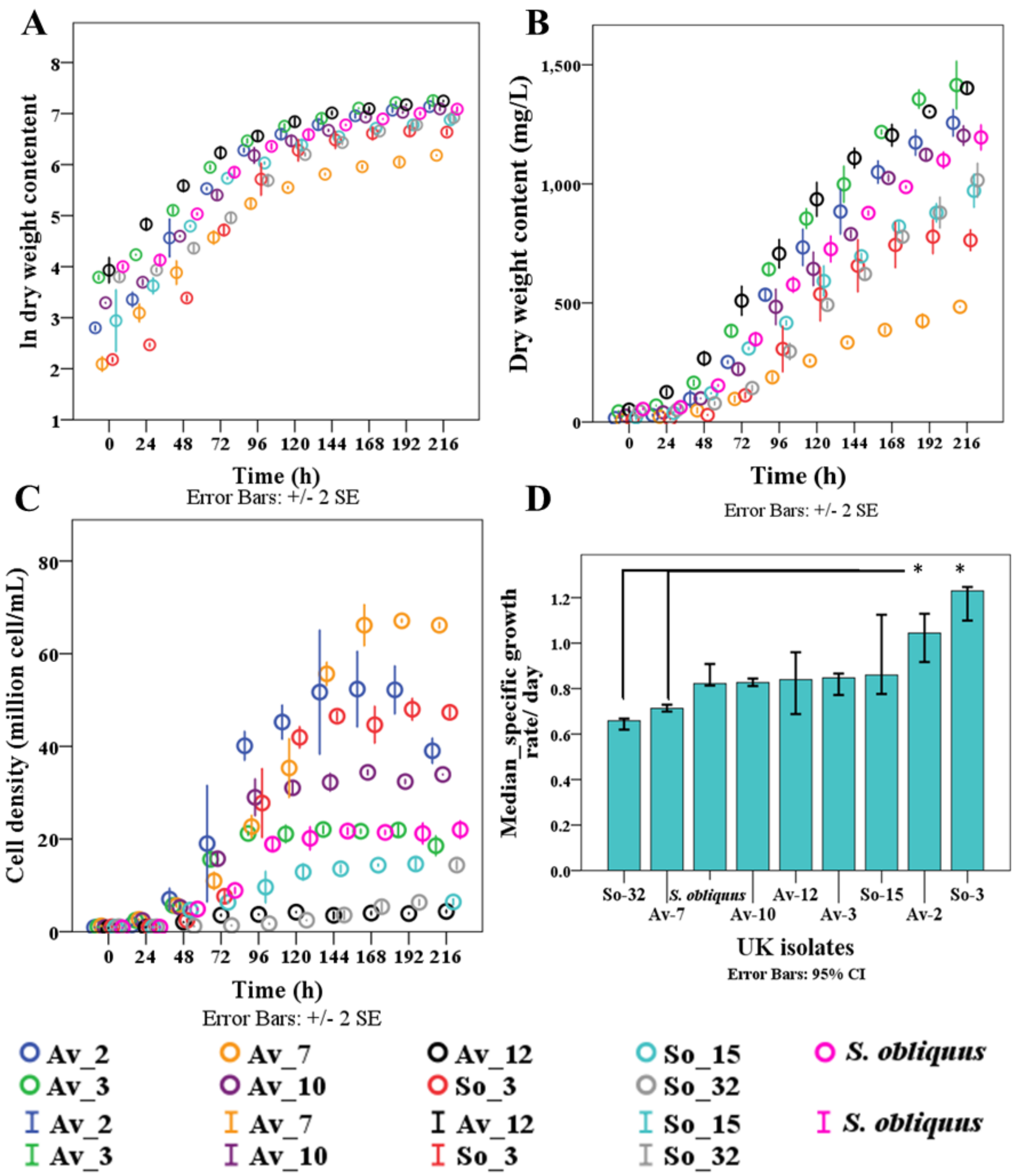

Figure 4.1: Growth assessment of UK-isolates and the reference strain (*S. obliquus*) in Somerton WW. Algae isolates were grown as triplicate in batch cultures under the following conditions: light intensity 130-150 µmol·photon·m^{-2}·s^{-1}, L:D cycle in hours 16:8, at 22 °C, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm for 10 days. **(A)** The growth curve is based on the dry weight (error bars represent standard error; $n = 3$). **(B)** Biomass accumulation during the experiment (error bars represent standard error; $n = 3$). **(C)** Cell density of the cultures during the experiment (error bars based on the standard error; $n = 3$). **(D)** Median specific growth rate for isolates and reference. Kruskal-Wallis test followed by homogenous subsets analysis based on asymptotic significances (Error bars represent 95% confidence intervals; asterisks indicate significant differences between UK isolates; $p < 0.05$).

Jo-29 is the fastest growing microalgae among Jordan isolates in WW of Jordan.

The growth of the microalgae isolates indigenous to Jordan was evaluated. The WW character in Jordan is different to that of Somerton. It is more concentrated and rich with phosphate, nitrate and ammonium (section 2.1.2 Table 2.4). The character of the WW used in this experiment is similar to the average of the final effluents from WWTPs in Jordan in 2013. For the UK isolates we used the secondary effluent obtained from Somerton WWTP. The characters of Somerton WW secondary effluent were modified by the addition of phosphate, nitrate and ammonium. This modified WW was seeded with the microalgae isolates from Jordan and the reference *S. obliquus* CCAP 267/7. Isolates were grown in batch cultures with three replicates in the optimum conditions in the growth room with CO_2 addition and mixing (section 2.1.3 and 2.2.14) for 10 days until they began the early stationary phase (figure 4.2 A). Growth was monitored by a daily measurement of DW, cell count and OD 750 nm.

At the end of the experiment Jo_29 accumulated the highest biomass (1678 mg/L) followed by Jo_2 (1391 mg/L). The lowest biomass produced was from Jo_40 (Figure 4.2 B). While Jo_4 had the highest cell count (54×10^6 cell/mL) followed by Jo_29 and Jo_18 with ca. 36×10^6 and 38×10^6 cell/mL respectively (Figure 4.2 C), the lowest cell count was for Jo_2 and Jo_12: ca. 15×10^6 and 16×10^6 cell/mL respectively.

The specific growth rate was calculated based on the daily dry weight measurements. Since the data were not normally distributed, results were analysed using Kruskal-Wallis Test, followed by homogenous subsets analysis based on asymptotic significance (p = 0.05). There was a significant difference between the medians of the specific growth rates of the samples. Jo_29, which has the highest specific growth rate value is significantly different from Jo_40, which has the second highest specific growth rate value and the rest of the samples including the reference. Jo_40 also has a significantly higher specific growth rate value than the rest of the isolates including the reference strain. In contrast, Jo_2 has the lowest specific growth rate value (Figure 4.2 D and Table 4.2 B). The specific growth rate values, generation time and biomass production data after 9 days are presented in Table 4.2 B.

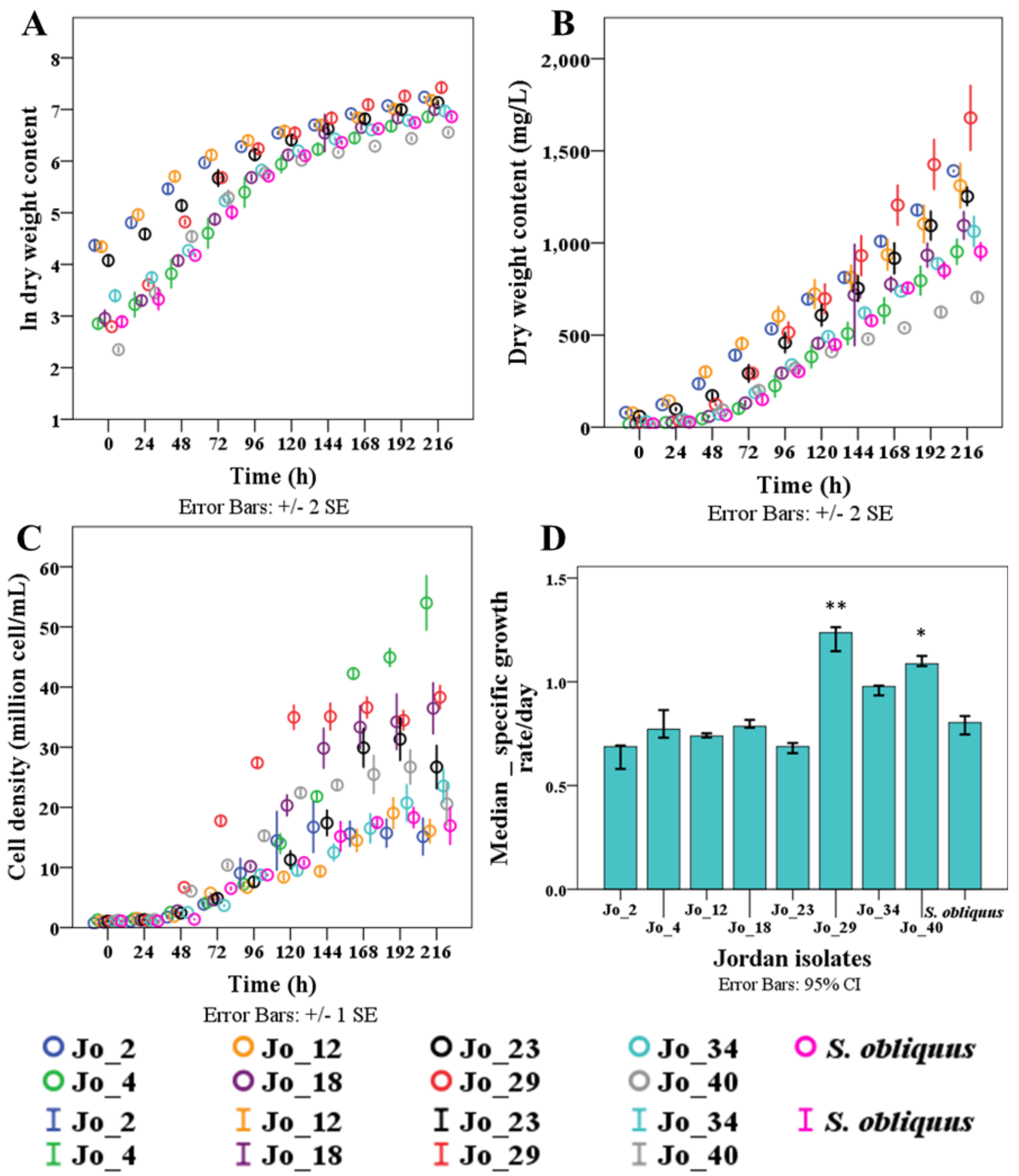

Figure 4.2: Growth assessment of Jordan isolates and the reference strain *S. obliquus* in average final effluent WW in Jordan. The algae isolates were grown in triplicate batch cultures at the following conditions: light intensity 130-150 µmol·photon·m^{-2}·s^{-1}, L:D cycle in hours 16:8, at 22 °C, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm for 10 days. **(A)** Growth curve based on the ln dry weight from daily measurement (error bars show mean standard error; n = 3). **(B)** The actual dry weight (biomass) values (error bars show mean standard error; n = 3). **(C)** The cell density measurements (error bars show mean standard error; n = 3). **(D)** Median μ values for isolates and reference. Kruskal-Wallis test followed by homogenous subsets analysis based on asymptotic significances revealed that there was a significant difference among the median μ values. (error bars represent the confidence intervals level at 95% n = 3; asterisks indicate the significant difference between the isolates and the reference strain; $p < 0.05$).

4.3.2 Capacity of microalgae to remove nitrogen and phosphorus

For WW treatment processes using microalgae, it is advantageous to apply microalgae that are able to remove nutrient such as nitrogen and phosphorus in the shortest time possible for an efficient process. This section evaluates nutrient removal from WW by microalgae isolates from the UK and Jordan.

The majority of the UK isolates removed nitrogen as well as the reference strain

Here, nitrogen removal by the UK isolates from Somerton WW secondary effluent is evaluated. A daily measurement of nitrate, ammonium and total nitrogen was taken until the samples reached below the detection level. In Somerton WW secondary effluent, most of the nitrogen is in nitrate form (section 2.1.2 Table 2.4)**.** Ammonium was present at a concentration of less than 0.5 mg/L and was consumed by most of the isolate after 24 h except for Av-2, for which after 72 h reached below the detection level (0.02 mg/L NH_4-N; Figure 4.3 A). Nitrate—the most available form of nitrogen—was removed until it reached below the detection level (0.5 mg/L NO_3-N) after 96 h (Figure 4.3 B). The total nitrogen test was performed to detect both organic and inorganic nitrogen in the WW, and was taken daily (Figure 4.3 C). Av_2 had the highest percentage of nitrogen removal 94.24 % among the isolates and the reference. The lowest percentage of removal was 85.26% for So-15. Percentage nitrogen removal in the samples are presented in Table 4.3 A.

The efficiency of the microalgae isolates in removing nitrogen during the experiment was assessed in order to rank them later. Equation 6 in section 2.2.16 was used to evaluate the specific nitrogen removal rate by the microalgae. To do that, two points were chosen whilst the algae were in the exponential phase and in the same time before the depletion of nitrogen from the WW. Specific nitrogen removal rates are shown in Table 4.3 A. The reference strain *S. obliquus* CCAP 267/7 has the highest specific nitrogen removal (0.113 /day), meaning 0.113 mg/L of nitrogen removed by 1 mg/L DW equivalent of *S. obliquus* during the exponential phase per day. The lowest specific nitrogen removal rate was for So_32 (0.038 /day). One-way ANOVA with Tukey's *post-hoc* test was used determine significant differences for the nitrogen removal among the microalgae isolates and the reference. Av_2, Av_10, Av_12, So_3 and So_15 behaved similarly to the reference strain with regards to nitrogen removal. Meanwhile, So_32, Av_3 and Av_7 were not as efficient as the reference strain at removing nitrogen (Figure 4.3 D).

Jo-18 has the highest nitrogen removal rate among the isolates of Jordan

In the same manner as the UK isolates, the nitrogen removal from the WW of Jordan by Jordan microalgae isolates was evaluated in this section. As mentioned previously the WW of Jordan is more concentrated than that of Somerton. The nitrogen content is ~1.5 X the nitrogen content in Somerton WW (section 2.1.2 Table 2.4). It also has higher ammonium content, and although it took longer for the algae to remove the nitrogen, they removed it efficiently (Table 4.3 B). The microalgae started to uptake ammonium because it requires less energy, as it is a ready to uptake form (Delgadillo-Mirquez et al. 2016). Most of the ammonium under these experimental conditions were removed after 120 h (Figure 4.4 A). The average percentage for ammonium removal for the all isolates after 120 h was 99.7%. While the nitrate consumption started after 48 h and by the 9^{th} day (192 h) more than 98% of the total nitrate was removed (figure 4.4 B), the percentage of total nitrogen removal under optimal conditions at the end of the experiment ranged from 89.47% for Jo_40 to 97.5% for Jo_4 (Figure 4.4 C and Table 4.3 B).

The above numbers give a general idea of the algal isolates able to remove the nitrogen from the WW under those conditions, but do not indicate which are faster in removing the nitrogen. To rank the different isolates depending on removing nitrogen from the WW, the specific nitrogen removal rate per day has been calculated according the same equation mentioned above (Equation 6 in section 2.2.16), by taking two points at the exponential phase and before the nitrogen depletion from the WW. The results are shown in Table 4.3 B. Jo_18 was the fastest at nitrogen removing, with. 0.0948 mg/L of nitrogen removed by 1 mg/L DW equivalent of Jo_18 in their exponential phase per day, whereas Jo_2 was the slowest isolate with a specific nitrogen removal rate per day of 0.016/ day. After data analysis using one-way ANOVA and Tukey's *post-hoc* test, Jo_18 removed more nitrogen than *S. obliquus,* while the rest of the isolates except Jo_2 were as efficient as the reference strain in removing the nitrogen from WW (Figure 4.4 D).

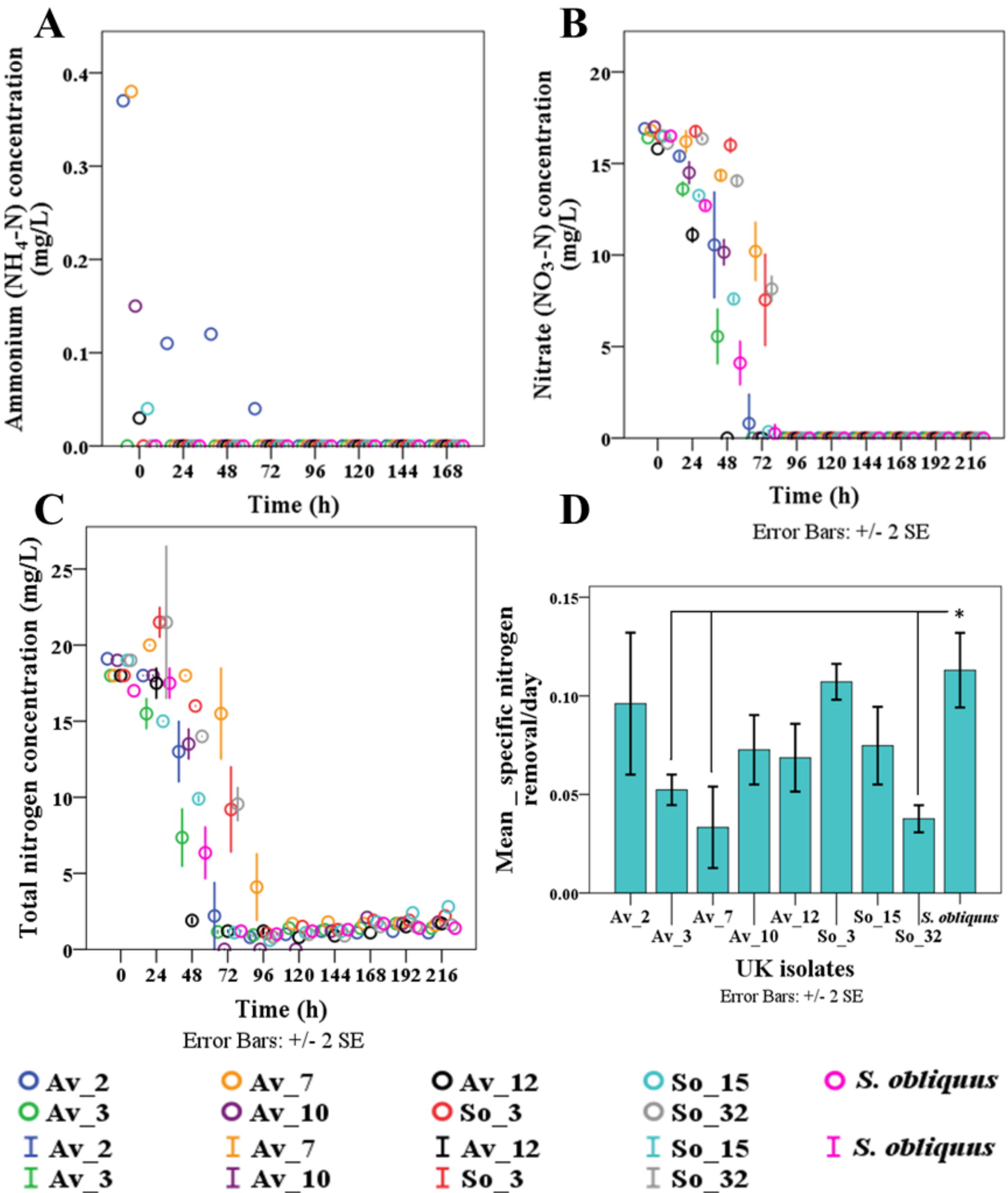

Figure 4.3: Nitrogen removal from Somerton WW secondary effluent by UK-isolates and *S. obliquus* CCAP 267/7. Samples were grown in triplicate under the following conditions: light intensity 130-150 µmol·photon·m^{-2}·s^{-1}, L:D cycle in hours 16:8, at 22 °C, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm for 10 days. **(A)** Ammonium removal from the WW: the ammonium concentration was very low in WW, so only one represented sample was measured from each isolate. **(B)** Nitrate removal during the experiment time, nitrate was the major nitrogen source in the WW. Most of it goes below the detection level after 120 h. **(C)** Total nitrogen removal from WW, most of the nitrogen was removed after 120 h. **(D)** Mean specific nitrogen removal rate among the UK isolates (asterisk indicates significant difference between the reference and the UK isolates; $p < 0.05$; one-way ANOVA and Tukey's *post-hoc* test; error bars represent standard error; $n = 3$). There are no error bars on the measurement at $t = 0$ because only one replicate was measured.

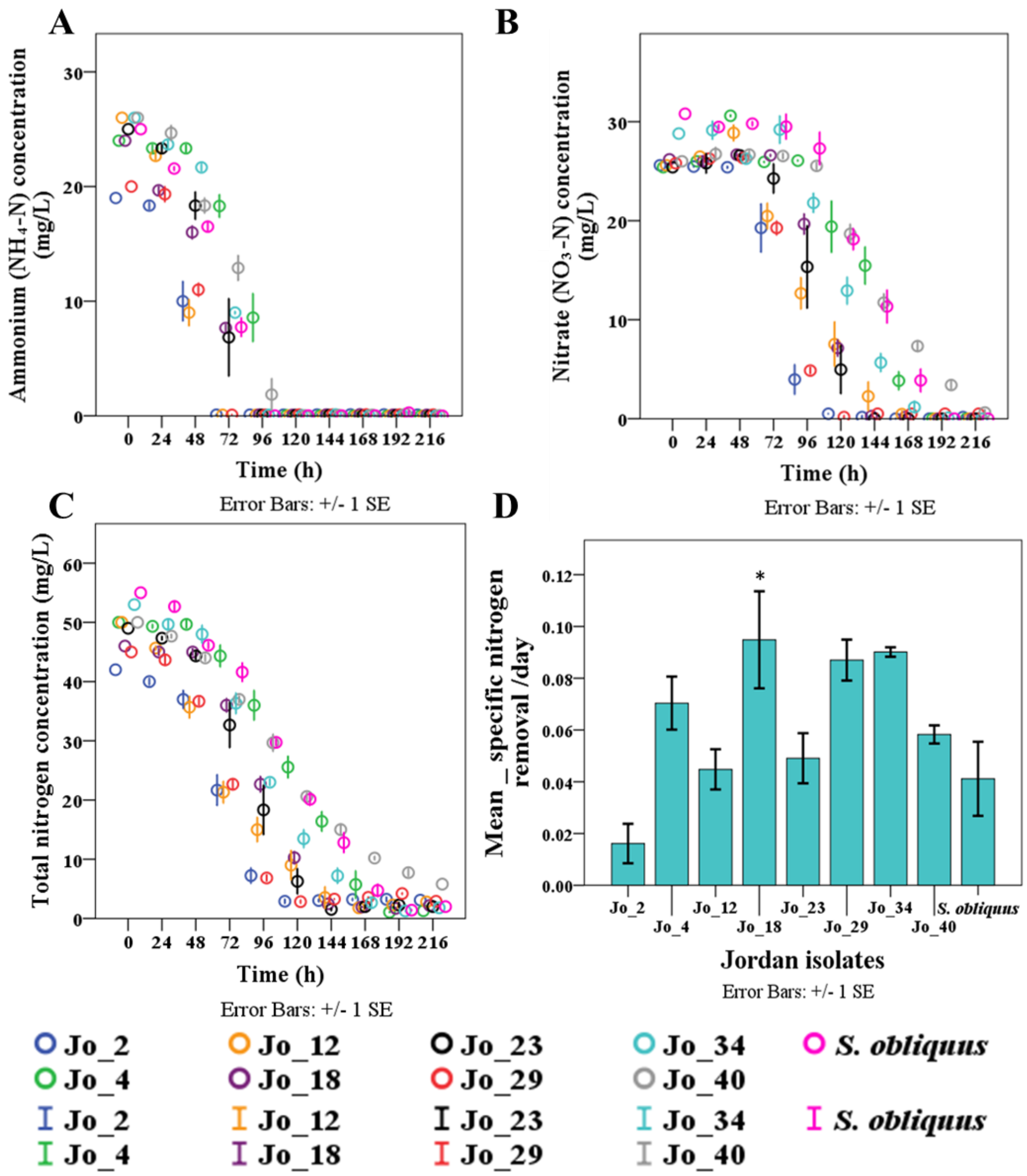

Figure 4.4: Nitrogen removal from the final effluent of artificial Jordan WW by the Jordan isolates and *S. obliquus* CCAP 267/7. Samples were grown in triplicate under the following conditions: light intensity 130-150 μmol·photon·m^{-2}·s^{-1}, L:D cycle in hours 16:8, at 22 °C, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm for 10 days. **(A)**. Ammonium removal, after 120 h the ammonium was totally removed by the microalgae. **(B)** Nitrate removal throughout the experiment, nitrate consumption started after 48-72 h and by the end of the experiment most of it was removed **(C)** Total nitrogen (organic and inorganic form) removal by the microalgae, by the end of the experiment most of the nitrogen has been removed. **(D)** Mean specific nitrogen removal rate per day for each of the microalgae samples. One-way ANOVA with Tukey's *post-hoc* test was used to evaluate the difference between the isolates (Asterisk indicates significant difference between Jo_18 and the reference strain; $p < 0.05$; error bars are based on standard error; $n = 3$). There are no error bars on the measurement at $t = 0$ because only one replicate was measured.

Table 4.3: Average specific nitrogen removal rate, % nitrogen removal from WW. (A) Data for nitrogen removal by the UK isolates, (B) data for nitrogen removal by Jordan isolates.

A			B		
UK isolates	**N removal /day**	**% N removal**	**Jordan isolates**	**N removal /day**	**% N removal**
Av_2	0.096	94.24	**Jo_2**	0.016	92.62
Av_3	0.052	92.22	**Jo_4**	0.070	97.53
Av_7	0.033	91.11	**Jo_12**	0.045	94.40
Av_10	0.073	90.53	**Jo_18**	0.095	95.33
Av_12	0.069	90.56	**Jo_23**	0.049	95.65
So_3	0.107	87.78	**Jo_29**	0.087	93.56
So_15	0.075	85.26	**Jo_34**	0.090	96.73
So_32	0.038	91.58	**Jo_40**	0.058	89.47
S. obliquus	0.113	91.76	***S. obliquus***	0.041	96.48

Among the UK isolates So-15 was the fastest at removing phosphate from WW

The efficiency of the UK microalgae isolates for phosphate removal from Somerton WW was evaluated. The major form of phosphorus in WW effluent is phosphate (PO_4^{3-}; Dueñas et al. 2003; Wei et al. 2008). Somerton secondary effluent WW batch had 2.37 mg/L of PO_4-P (section 2.1. 2 Table 2.4). During 10 days of growth under optimum conditions with CO_2 supplementation (section 2.1.3 and 2.2.14), the 8 microalgae isolates and *S. obliquus* were able to remove almost all phosphate, until it reached below the detection level (0.05 mg/L PO_4-P). After 96 h, most of the phosphate was removed from the WW (Figure 4.5 A). By the end of the experiment the % removal of phosphate for all the isolates was 100 % Table 4.4 A.

To assess how efficient the microalgae isolates were in removing the phosphate, the specific phosphate removal per day was calculated from two points at the exponential phase and before phosphate depletion from the WW using equation 3 section 2.2.16. Table 4.4 A presents the values of the specific phosphate removal rate. To detect the difference between the microalgae isolates in phosphate removal behaviour, a Kruskal-Wallis test was done followed by homogeneous subsets based on asymptotic significances $p < 0.05$ (Figure 4.5 B). The results of this analysis showed that So_15 is the fastest in removing phosphate from WW, in which 0.39 mg/L of phosphate are removed by the So-15 culture equivalent to 1 mg/L of DW in the exponential phase per day, followed by So_3 with a specific phosphate removal of 0.024/day. The slowest isolate was Av_7 (0.009 /day), which behaved in a similar way to the reference *S. obliquus* (0.010 /day; see Figure 4.5 B and Table 4.4 A).

The majority of the Jordan isolates removed the phosphate as well as the reference strain

Phosphate removal by the microalgae isolates obtained from Jordan was assessed. Phosphate concentration in the modified WW was ~5.8 mg/L PO_4-P (section 2.1. 2 Table 2.4). Under the conditions of the experiment most of the phosphate was removed after 120 h by all the isolates except Jo-4 (Figure 4.6 A). Table 4.4 B shows the percentage phosphate removal by the microalgae isolates by the end of the experiment. This ranged from 97.46% (Jo-2) to 99.66% (Jo-23). To assess how fast the isolates were in removing phosphate, the specific phosphate removal rate was calculated from two time points at the exponential phase and before phosphate depletion, in a similar way as for the UK isolates (equation 3 section 2.2.16). The highest specific phosphate removal value was for Jo-40 (0.0179 /day) followed by Jo-18 (0.0177 /day), and the lowest value was for Jo-2 (0.003 /day) as presented in Table 4.4 B. The statistical analysis for these values by one-way ANOVA and Tukey's *post-hoc* test ($p < 0.05$) showed all the isolates act like the reference strain *S. obliquus* except Jo-2, which was slower to remove phosphate (Figure 4.6 B).

Table 4.4: Average specific phosphate removal rate and percentage phosphate removal from WW. (A) Data for phosphate removal by UK isolates, (B) data for phosphate removal by Jordan isolates.

A			B		
Isolates	PO_4^{3-} removal /day	% PO_4^{3-} removal	Isolates	PO_4^{3-} removal /day	% PO_4^{3-} removal
Av_2	0.017	100	**Jo_2**	0.00318	97.46
Av_3	0.012	100	**Jo_4**	0.01192	99.42
Av_7	0.009	100	**Jo_12**	0.01262	99.54
Av_10	0.011	100	**Jo_18**	0.01773	97.82
Av_12	0.011	100	**Jo_23**	0.00759	99.66
So_3	0.024	100	**Jo_29**	0.00587	97.08
So_15	0.039	100	**Jo_34**	0.01280	98.95
So_32	0.012	100	**Jo_40**	0.01795	97.48
S. obliquus	0.01	100	***S. obliquus***	0.01601	99.05

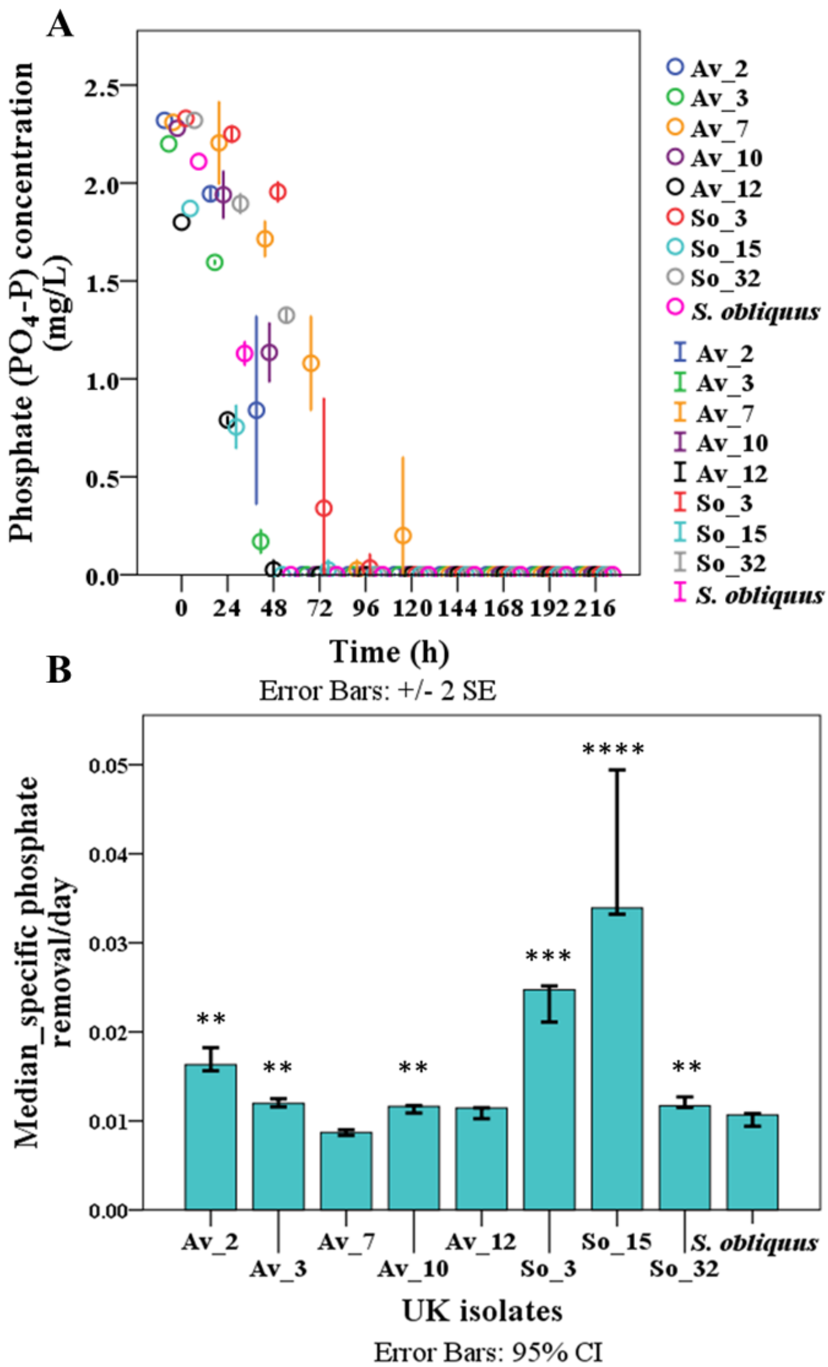

Figure 4.5: Phosphate removal by UK isolates from Somerton WW. Samples were grown in triplicate under the following conditions: light intensity 130-150 µmol·photon·$m^{-2}·s^{-1}$, L:D cycle in hours 16:8, at 22 °C, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm for 10 days. **(A)** Behaviour of UK isolates and reference strain in removing phosphate from Somerton secondary effluent. During the experiment, most of the phosphate was removed after 96 h (error bars represent SE; n = 3; there are no error bars on the measurement at t = 0 because only one replicate was measured). **(B)** Median of the specific phosphate removal rate for UK isolates. (Asterisks indicate significant difference from the reference strain analysed by Kruskal-Wallis test followed by homogenous subset analysis; p < 0.05; error bars represent 95 % confidence intervals; n = 3).

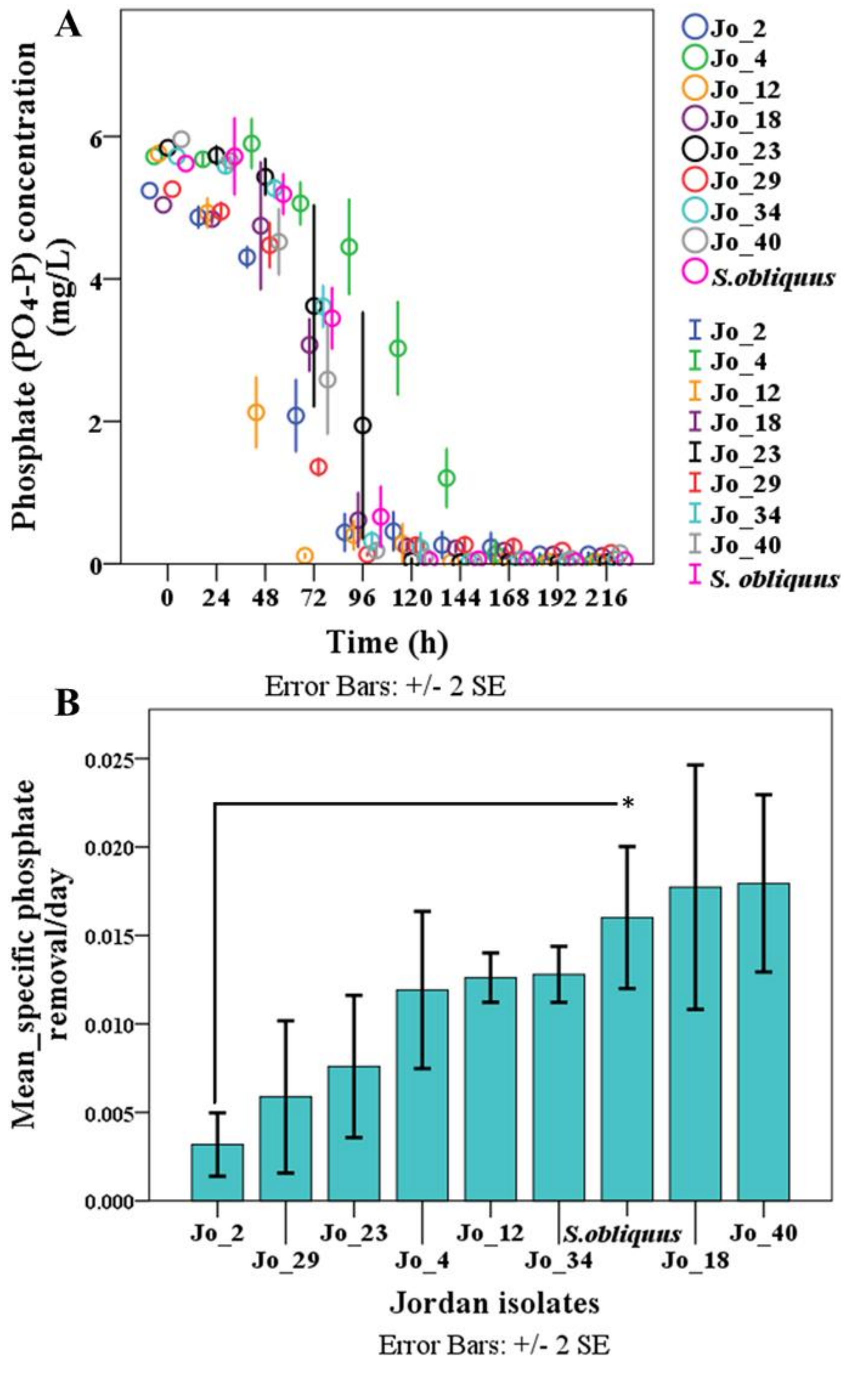

Figure 4.6: Phosphate removal from WW by microalgae isolates obtained from Jordan. Samples were grown in triplicate under the following conditions: light intensity 130-150 µmol·photon·m^{-2}·s^{-1}, L:D cycle in hours 16:8, at 22 °C, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm for 10 days. **(A)** Phosphate removal by Jordan isolates, most of the phosphate was removed after 120 h. **(B)** Mean specific phosphate removal rate, all microalgae isolates removed phosphate as well as the reference except Jo_2 (one-way ANOVA with Tukey's *post-hoc* test; $p < 0.05$; error bars represent standard error; $n = 3$). There are no error bars on the measurement at $t = 0$ because only one replicate was measured.

4.3.3 Assess Algal settleability

As mentioned in section 1.1.4 harvesting microalgae from a large-scale culture is a major challenge since it increases the cost of the WW treatment process. Harvesting microalgae by sedimentation or settling by gravity offers a simple and low cost option for microalgae harvesting. This section evaluates the settleability of microalgae by gravity.

Somerton_3 was the fastest microalgae isolate to settle by gravity

Microalgal settleability has been assessed by measuring the sludge volume index (SVI) and the percentage of settled cells at different time points (0.25, 0.5, 1, 2, 3, 4, 8 and 24 h). SVI is a common method used in WWTPs to describe the settling characteristics of sludge. It is the volume (mL) occupied by one gram of sludge after settling for 30 minutes. SVI is measured by an Imhoff cone or 1L graduated cylinder (equation 7, section 2.2.17). The Imhoff cone is filled with mixed liquor was sampled from the aeration tank and allowed settle for 30 minutes. An SVI value of around 50 mL/g indicates very good settling and if the value is more than 120 mL/g, it indicates poor settling characteristics (EPA, 1997). As a consequence, if the SVI values for algae are around 50 mL/g this means better settling characters and a lower cost for harvesting.

Settling experiments were conducted in 500 mL glass bottles and the phase separation was monitored and measured to calculate the SVI. The microalgal cells are small and do not settle by gravity as fast as other suspended solids in the WW. Thus, the SVI values were very high, the lowest SVI value obtained was for Av-12 after 24 h settling (140 mL/g), then So_3 (275 mL/g), which is still considered high (poor settling character). However, the highest value of SVI after 24 h settling was for Av_7 (27850 mL/g) indicating very poor settling. Mid-way settling time after 8 h has been chosen to assess the settleability of the UK algae isolates (Figure 4.7 A), in which So-3 has the lowest SVI value at that time (275 mL/g) followed by Av-12 and Av-10. The highest SVI value after 8 h was for AV_7 (~28000 mL/g) followed by *S. obliquus* and Av_2 (~13800 mL/g and ~13500 mL/g respectively). Statistical analysis to evaluate the differences in settleability depending on the SVI was assessed by a Kruskal-Wallis test followed by homogenous analysis based on asymptotic significances at the 0.05 significance level. The result showed a significant difference in settling behaviour only between So_3 and Av_7 (Figure 4.7 B).

The percentage of the settled cells at 1 cm depth has been calculated at different time points (Figure 4.7 C). After 8 h, the highest percentage of settled cells was for So_3 (82.6%) followed by Av_12 and Av_10 (73.6% and 62% respectively) and the lowest was for Av_7 (8.35%) as was the case for the SVI. Statistical analysis of the mean percentage of settled cells using one-way ANOVA and Tukey's *post-hoc* test showed that there was only a significant difference between So_3 and Av_7 after 8 h. It is worth noticing that the behavior of the algae was not consistent, it behaved differently between the two trials. This may be the reason a significant different was not observed between the microalgae isolates.

Jordan- 12 was the fastest to settled by gravity

To evaluate the settleability of the Jordan isolates, the settling experiment was performed in the same manner as it was done for the UK isolates. In a 500 mL glass bottle, 400 mL of algal culture in the log phase with approximately similar cell count was done in two trials (section 2.2.17). The SVI and percentage of settled cells has been obtained at different time points: 0, 0.25,0.5, 1, 2, 3, 4, 6, 8 and 24 h (Figure 4.8 A). The results of the SVI ranged from good to poor settleability. Figure 4.8 B shows the SVI results at 0, 8 and 24 h. Jo_12 has the lowest SVI (38 mL/g) after 24 h of settling, followed by *S. obliquus*, J_34 and Jo_2 (54 mL/g, 75.6 mL/g, 75.6 mL/g respectively). Jo_12 has SVI lower than 50 mL/g, indicating good settling character. The highest SVI value was for Jo_40, followed by Jo_23 (9570 and 2060 mL/g) these values indicate poor settleability character. Statistical analysis for the SVI values after 8 h of settling by Kruskal-Wallis test for all the Jordan isolates and the reference showed no significant difference between these isolates. However, we can see a difference in the behaviour (Figure 4.8 A), which may be due to the fact that there were only two replicates, and sometimes fluctuation in the SVI value between the first and the second trial. This will be addressed in the discussion. The percentage of settled cells at 1 cm depth was measured after 24 h. The highest percentage of settled cells was for Jo_12 (97%) and Jo_2 (94%). The lowest percentage was for Jo_40 with only 28% of cells settled. Figure 4.8 C presents the percentage settled cells at 0, 8 and 24 h. Unfortunately, there was missing data for Jo_40 at t = 8 and 24 h, this is the reason there were no error bars at the isolate in figure 4.8 C. Statistical analysis of the samples using Kruskal-Wallis test showed no significant differences between the samples after 8 h of settling and this is may be due to the low number of replicates will not be enough for a robust statistical analysis.

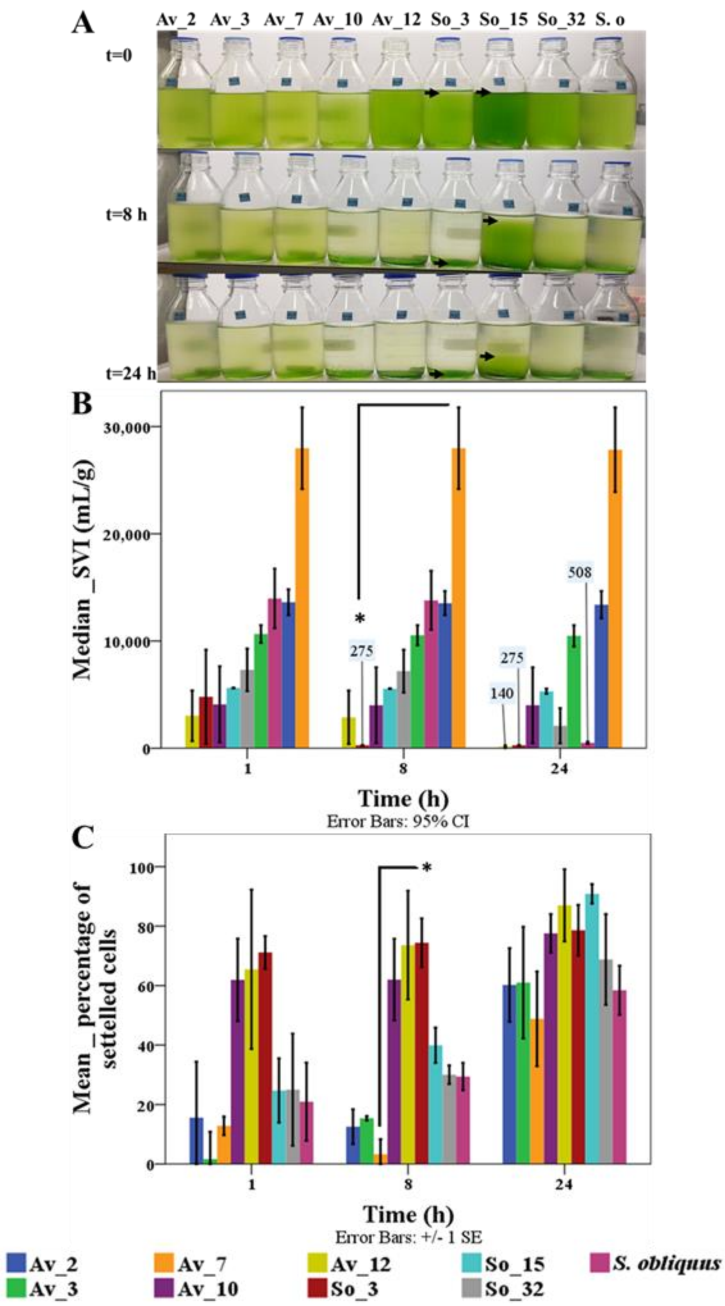

Figure 4.7: Settleability by gravity of the UK algal isolates. Samples were grown under the following conditions: light intensity 130-150 µmol·photon·m^{-2}·s^{-1}, L:D cycle in hours 16:8, at 22 °C, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm. The algae were in the log phase with cell density around 5×10^6 cells/mL. **(A)** Algal settling after 0, 8 and 24 h, the phase separation (clear phase and algal phase) is indicated by black arrows in some bottles. The height of algal settled phase was used to calculate the volume of settled algae for SVI values. **(B)** Median of the SVI values for UK isolates after settling for 8 h. Kruskal-Wallis test followed by homogenous subsets analysis based on asymptotic significances at 0.05 significance level shows that there is a significant difference indicated by the asterisk (error bars represent 95% confidence intervals). **(C)** Mean percentage settled cells at a fixed-point 1 cm depth from the surface measured at three different time points 1, 8 and 24 h. One-way ANOVA with Tukey's *post-hoc* test was performed to check for differences between isolates after 8 h, shown by the asterisk ($p < 0.05$ error bars represent standard error; $n = 2$).

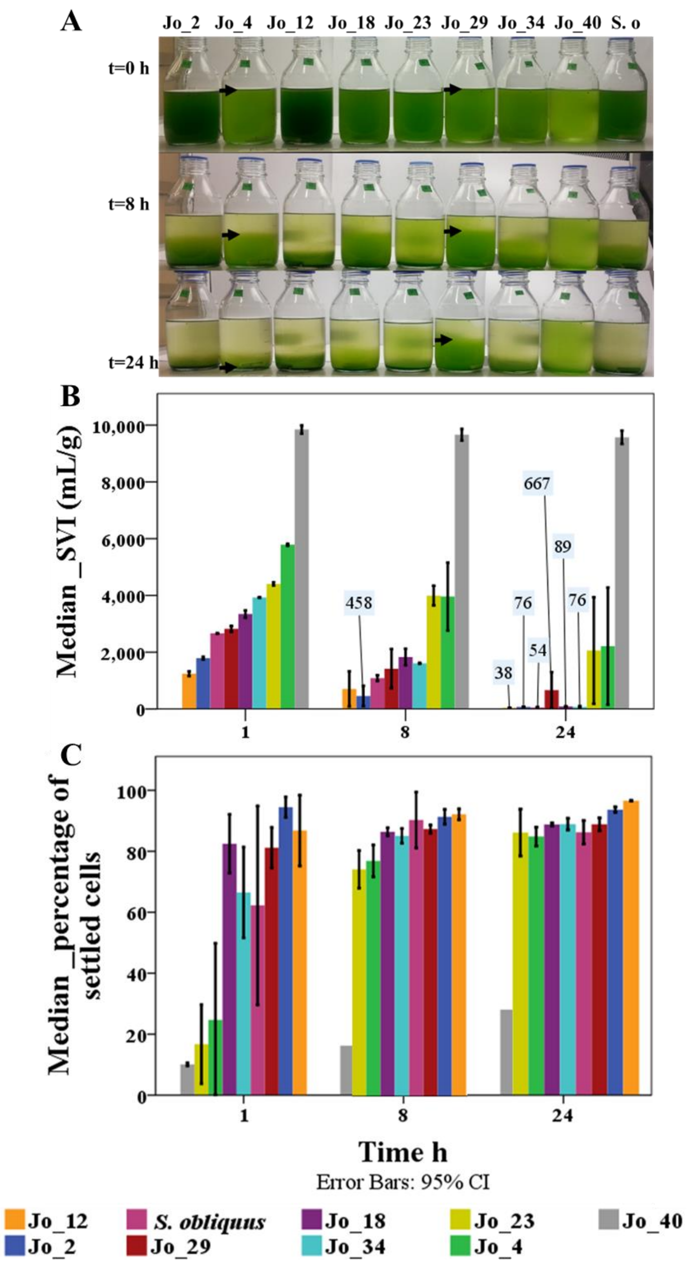

Figure 4.8: Settleability by gravity of Jordan isolates. Samples were grown under the following conditions: light intensity 130-150 µmol·photon·m^{-2}·s^{-1}, L:D cycle in hours 16:8, at 22 °C, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm. Algae in log phase with cell density ~6-7 × 10^6 cells/mL. **(A)** Algal settling after 0, 8 and 24 h, phase separation (clear phase and algal phase) indicated by black arrows in some bottles. The height of algal settled phase was used to calculate the volume of settled algae for SVI values. **(B)** Median SVI values for the Jordan isolates after settling for 1, 8 and 24 h. Kruskal-Wallis test followed by homogenous subsets analysis based on asymptotic significances at 0.05 significance level shows that there is no significant difference between the SVI values of the Jordan isolates after 8 h of settling. **(C)** The Median of the percentage of the settled cells at a fixed-point ca. 1 cm depth from the surface at three different time points 1, 8 and 24 h. Kruskal-Wallis test followed by homogenous subsets analysis based on asymptotic significances at 0.05 significance level shows no significant differences between the percentage of settled cells among the Jordan isolates after 8 h of settling (error bars represent confidence intervals at 95%; n = 2).

4.3.4 Overall evaluation of microalgae isolates performance

This research is looking for a microalga isolate that is adapted to the local environment and could be applied in the WWTPs for efficient nutrient removal and biomass production. This is the first and primary stage in evaluating the performance of microalgae isolates and choosing a candidate for upscaling. Some of the characteristics that have been assessed for the obtained microalgae isolates from the UK and Jordan are: growth rate, biomass productivity, phosphate removal, nitrogen removal and settling by gravity.

The obtained isolates from the UK and Jordan were given a rank 1-4. The best performance in any character will be given 1 and the worst 4. As a result of this ranking the lowest total score indicates a better rank than the rest of the isolates. The characteristics and the ranking scores are presented in Table 4.5 for the UK isolates, whilst Table 4.6 shows the ranking of the Jordan isolates. The characteristics of ranking are nitrogen and phosphate removal rate per day, growth rate and settleability which was given double points because it is a main characteristic of interest to lower the cost of harvesting, which is a major issue in applying algae for many purposes (Olguı´, 2003; De la Noue, Laliberte and Proulx, 1992).

Among the UK isolates So_3 performed the best in most of the characteristics with a total score of 6, the lowest obtained score. A problem encountered with this isolate was that the cells stick together and to the surface of the glassware and needs high-speed mixing to form a homogenous culture which could be difficult to apply in large scale cultures. The second candidates Av_12 and Av_10 performed well, especially in settling. Av_2 and So_15 have the same scores (11) but they have bad settling characteristics, which is a main issue in our case as mentioned above for cost reduction. Av_12 has been chosen to be scaled up to be grown in 8 L column photobioreactors and 500 L raceway pond in the glass house of the University of Bath. And then it was applied for a pilot scales HRAPs in Beckington WWTPs. Av_10 is another alternative if Av_12 fails to achieve the goal or applying both of them together can be an option.

Regarding the Jordan samples, the settleability of the microalgae has been given a double point as well as the UK-isolates (see Table 4.6). Jo_18 and Jo_29 has the lowest total scores (7) which means that they perform well in most of the characteristics. Both could be good candidates for upscaling in the future in Jordan. Jo_34 could also be a candidate.

It may worth noticing that Av-10 and Jo_29 are closely related and Av-12 and Jo-34 are the same species and the same as the reference *S. obliquus*, which they performed better than it (Figure 3.6). Although So-3 and Jo_40 are the same species but they behaved differently in the settling experiment.

Table 4.5: Ranking the UK isolates according to the studied criteria.

Microalgae isolate	Growth rate/ Productivity	PO_4^{3-} removal	N removal	Settleability (2X)	Total score
Avonmouth_2	1	3	1	3 (6)	11
Avonmouth_3	2	3	3	3 (6)	14
Avonmouth_7	3	4	3	4 (8)	18
Avonmouth_10	2	3	2	2 (4)	11
Avonmouth_12	2	3	2	2 (4)	11
Somerton_3	1	2	1	1 (2)	6
Somerton_15	2	1	2	3 (6)	11
Somerton_32	3	3	3	3 (6)	15
S. obliquus CCAP 276/7	2	4	1	3 (6)	13

The lowest score means the best performing green microalgae.

Table 4.6: Ranking the Jordan isolates according to studied criteria.

Microalgae isolate	Growth rate/ Productivity	PO_4^{3-} removal	N removal	Settleability (2X)	Total score
Jordan_2	4	4	4	1 (2)	14
Jordan_4	3	2	2	2 (4)	11
Jordan_12	3	2	3	1 (2)	10
Jordan_18	3	1	1	1 (2)	7
Jordan_23	4	3	3	2 (4)	14
Jordan_29	1	3	1	1 (2)	7
Jordan_34	3	2	1	1 (2)	8
Jordan_40	2	1	2	3 (6)	11
S. obliquus CCAP 276/7	3	1	3	1 (2)	9

The lowest score means the best performing green microalgae.

4.4 Discussion

The performance of the microalgae isolates in treating WW in batch cultures for 10 days has been evaluated for the UK and Jordan microalgal isolates. The evaluation was based on their growth in WW, nitrogen removal efficiency, phosphorus removal efficiency and the settleability by gravity for the candidates. After the evaluation process, there were two candidates from the UK with a promising performance (Av_12 and Av_10) and another two isolates from Jordan (Jo_18 and Jo_29). These isolates present good candidates for large-scale WW treatment experiments. In this section, the variation in the performance of the different microalgae isolates will be discussed.

4.4.1Growth rate and cell size

Specific growth rate was used for the assessment of the performance of microalgae in WW treatment. Among the UK isolates So_3 and Av_2 were the fastest in growing and Jo_40 was a fast-growing microalga too among the Jordan isolates, all of them belong to the genus *Chlorella* and it has smaller cell size than *Scenedesmus* or *Desmodesmu*s (Figure 3.3 and Figure 3.6). However, Jo_29 larger in size than Jo_40 which belong to the genus *Desmodesmus* has a higher growth rate than Jo_40.

The maximum growth rate and cell size have an inverse relation; small cells have higher growth rate than large cells (Tang, 1995; Bec et al. 2008). This is because small cells have thinner diffusion boundary layers and a larger surface area to volume ratio which enables them to aquire more nutrients under oilgtrophic conditions (Raven, 1998; Bec et al. 2008). But this kind of relationship could not be applied in all the cases. Smaller than certain sizes (2-3 µm) the growth rate decreases with decreasing size of the cells under both resource conditions when they are saturated or scarce. It has been suggested that this deviation from the relationship is due to constraints on size due to the limited necessary occupation space for the the large cellular biomass, and this could decrease the the growth rate of the cell (Bec et al. 2008).

4.4.2 Nutrient removal by microalgae

In this study, microalgae isolates were obtained from local environments (8 microalgae isolates from the UK and 8 from Jordan) and grown in WW in a batch culture system to demonstrate how efficiently they could remove nitrogen and phosphate from WW in comparison with a reference strain. After analysis of the results and ranking algae for nitrogen removal, *S. obliquus*, So_3 (*Chlorella sorokiniana*) and Av_2 (*Chlorella luteoviridis*) were the best among the UK isolates. Whereas Jordan Jo_18 (*Desmodesmus Sp.*), Jo_34 (*S. obliquus*) and Jo-29 (*Desmodesmus Sp.*) were the best at nitrogen removal among the Jordan isolates. In the case of phosphate removal, So_15 (*Monoraphidium or Ankistrodesmus*) had the highest specific phosphate removal rate, which was significantly different from the rest of the isolates, followed by So_3 (*Chlorella sorokiniana*). Among the Jordan isolates Jo_40 (*Chlorella sorokiniana*), Jo_18 (*Desmodesmus Sp.*), and the reference *S. obliquus* performed the best. Some isolates such as So_3 and Jo_18 performed well at both phosphate and nitrogen removal. However, it is not always the case in which the strain that is the faster in phosphate uptake is the faster in nitrogen uptake or vice versa (Table 4.6 and Table 4.5). There are some microalgae isolates that behave very well in one characteristic and not in another like Av_2, Jo_29 and the reference *S. obliquus.*

The reference *S. obliquus* has a much lower (0.041 /day) specific nitrogen removal rate in Jordan WW than that in the UK WW (0.113 /day). This may be due to the presence of ammonium and nitrate at relatively high concentrations, which may cause the system of nitrogen uptake in algae not to be stressed. UK WW has more limited nitrogen (less than the half that of WW of Jordan) so the system worked more efficiently because they felt more stressed.

Nutrient removal and cell size

Nutrient uptake as a physiological trait is affected by morphological and phenological traits as adaptive mechanisms to fit the environmental changes (Reynolds, 1984 cited in Sutherland et al. 2014). One of these factors is cell size, as it was argued that smaller cells are more efficient in removing nutrients than larger cells under low nutrient concentrations. Some studies have demonstrated that under oligotrophic conditions, size becomes relevant to nutrient removal, and as a result small phytoplankton dominate (Irwin et al. 2006; Naselli-flores and Barone, 2011). Since small cells such as prokaryotes have a large surface area to volume ratio, they have a larger surface for absorbing nutrients, and the nutrient can easily and rapidly distribute into the cells' interior. In addition, small cells have a smaller diffusion boundary layer that limits nutrient transport at least for spherical cells (Ploug et al. 1999; Naselli-flores and Barone, 2011).

Eukaryotic (large) cells have a smaller surface area to volume ratio, therefore nutrients are not rapidly diffused into the different interior parts of the cell. They therefore require different specialised organelles to provide energy and transport chemicals throughout the cell (Naselli-flores and Barone, 2011). The concentration of intracellular nutrients is higher than the concentration of nutrients in the environment, therefore nutrient uptake occurs against diffusion gradients with the help and control of sophisticated transport systems. The phytoplankton developed different approaches to optimise their nutrient uptake, for instance nitrogen fixation, production of alkaline phosphatase, mixotrophy and mucilage production, which facilitates nutrient sequestration and processing (Reynolds, 2007; Naselli-flores and Barone, 2011). Under experimental eutrophic conditions, the maximum phosphorus uptake rate increased with increasing colony and cell size. *Chlorella* as a small cell microalgae and *Microcystis* large colony microalgae both have a similar maximum phosphate uptake rate (Reynolds, 1993b cited in Naselli-flores and Barone, 2011).

In this study, the effect of cell size on the efficiency of nutrient removal was not investigated. WW is considered as a eutrophic environment, and this will minimise the cell size effect. For instance, So_15 (large cell size) did better than So_3 (small cell size) in the removal of phosphate. Jo_18, which has a colony form of 4 cells performed as well as Jo_40, which is small solitary *Chlorella* cell in phosphate removal, and it was better in nitrogen removal.

Nutrient removal and mixing

In this experiment, the batch cultures were subjected to continuous mixing which may have helped to sustain the large algal cells and colonies in the water column and provided them with the proper light needed for photosynthesis, and improved nutrient removal and biomass production. And not only the large algal cells, So_3 is *Chlorella sorokiniana* has small cells but it tends to clots and stick to the glass without a proper mixing it was not easy to obtain a homogenous and dense culture. This is a problem when they will be grown in large scale where a strong mixing is required. Sutherland and colleagues (2014b) performed an experiment to investigate the effect of changing the frequency of mixing on microalgae performance in nutrient removal, productivity and physiological adaptation for three morphologically different microalgae (*Chlorella vulgaris, Mucidosphaerium pulchellum, Pediastrum boryanum*) in an HRAP for WW treatment. They demonstrated that the efficiency of nutrient removal per unit of biomass and the biomass increased by increasing the frequency of mixing. Increased mixing frequencies and improved photosynthesis efficiency at low light sustain larger and denser colonies in the water column rather than settling down. Decreased mixing frequencies contributed to a lower efficiency of nutrient uptake from WW (Sutherland et al. 2014b).

4.4.3 Settling characteristic

In this study, our algae were seeded for settling experiments in their log phase in WW because we expect to apply them for WW treatment in the exponential phase and harvest them. Among the UK isolates So_3, Av_12 and Av_10 performed the best. Whereas, in Jordan isolate Jo_12, J_34 and Jo_2 were good in settling by gravity. There were many limitations affecting the results of the settling experiment, especially the SVI values.

The first limitation was in applying the method of SVI to assess the settling of microalgae that may cause inaccurate evaluation. That SVI method depends on measuring the volume of the settled algae as a result of expected two phase separations (clear phase for WW and settled algal phase). In some cases, there was no clear phase separation; the algal cells settled directly to the bottom but there is no clear phase separation. Another point is that the phase is not uniform in height or cell density and this may lead to inaccurate sludge volume estimation. Furthermore, some algae stick to the wall of the bottles and do not settle down. Moreover, algal settling is easily disturbed by liquid turbulence due to photosynthesis bubbling, or the sampling process. Sometimes you can see algae re-floated up.

The second factor affecting the results is the variation in the inoculated biomass. It was attempted to seed the WW equally in each group of isolates depending on the cell count and this caused a variation in the biomass. In the UK isolates, SVI values were higher than for the Jordan isolates even for So_3 which settled very fast (within less than 30 minutes), and the reason for these high values is that the initial started biomass for the UK isolates was lower than for the Jordan samples. Depending on the equation for SVI where it is divided by the biomass and there is an inverse relationship (equation 7, section 2.2.17), even if the UK and Jordan samples have the same settled sludge volume to sample volume ratio at the same time point, they would have different results depending on the initial biomass. The big difference in *S. obliquus* inoculated biomass between the UK (347.5 mg/L) and Jordan settling experiments was ~73 mg/L, which caused it to settle rapidly in the Jordan experiment. Whilst it settled slowly in the UK experiment the same effect has been noticed on Av_12 (*S. obliquus)*. In addition to the previous point, increasing the cell density for large cells like *S. obliquus* will increase the settling velocity in contrast to small cells like *Chlorella*. It has been observed that cultures with higher biovolumes (such as *Pediastrum/ Desmodesmus sp*.) have a higher theoretical and experimental settling velocity in HRAPs (Park, Craggs and Shilton, 2011b).

The third factor affecting the settling experiment was recycling the biomass, that is after settling for the first day the bottles have been vigorously mixed and set the same bottles for the second trial to be consistent. Except for So-3, because it formed something like a biofilm so it was hard to return it to the original suspended state. There were unexpected influences of recycling on the settling characters for some samples. It has been noticed that some samples slowly settled in the first trial but in the second trial it settled rapidly like Av_10 (*Desmodesmus sp.*), So_32 (*Scotiellopsis reticulata*), Jo_4 (*Desmodesmus sp.*) and Jo_23 (*Desmodesmus sp.*). It has been reported that recycling the algal biomass improves the settling characteristic and biomass productivity in HRAPs (Park Cragss and Shilton, 2015) and this could be due to the fact that recycled algae have a larger colony size. In contrast, Jo_29 in the second trial after recycling the biomass was slower in settling, behaving in the opposite way.

Finally, it is worth mentioning that in this experiment there were only two replicates. The behaviour of the microalgae isolates was sometimes different between the first and the second experiment due to factors mentioned above. Thus, the statistical analysis of the obtained data was not robust. For example, for the Jordan isolates there was no significant difference between the SVI values after 8 h, for Jo_12 (86 mL/g) and Jo_40 (9869 mL/g). The same was the case for the percentage of settled cells: no significant difference between Jo_12 (92.7%) and Jo_40 (16.2%). For all the above-mentioned reasons, it recommended improving this method of settling assessment by addressing all the abovementioned problems case by case.

Among the factors that affect the settling of microalgal cells is their morphology. Morphology varies between and within species and it widely depends on the environmental factors as it was discussed in chapter 3. Microalgal morphology affects the drag coefficient and buoyancy of the cells during settling (Manheim and Nelson, 2013). It has been reported that the presence of spines in *Scenedesmus* (may be identified now as *Desmodesmus*) could decrease the settling rate (Conway and Trainor, 1972 cited in

Manheim and Nelson, 2013). Another factor that was shown to affect algal cell settling is the cell size. The settling velocity for several green microalgae, cyanobacteria and diatom species has been studied, and it was reported that algae settling velocities increased with algal size and age (Titman and Kilham, 1976; Choi et al. 2006). Manheim and Nelson (2013) reported in their study for a species from *Scenedesmus* and *Chlorella vulgaris* that *Scenedesmus sp*., which has larger cells, settles faster than *Chlorella vulgaris* at the same physiological conditions.

These results agreed with some of our findings that in the Jordan isolate where Jo_40 *Chlorella sorokoniana* was the slowest in settling and it has small cell size. In the UK isolates Av_12 (*S. obliquus*) has large cells and forms colonies of four cells and is one of the best performing isolates in settling. In contrast, So_3 (identified as *Chlorella sorokinia*), which has solitary and small cells, performed the best in settling. This may be due to the characteristics of the cell surface such as the charge or the possibility of secreting material that promotes clotting and increases their settling velocity. Small microalgae usually flocculate to increase their size and density. Microalgae are able to produce some chemicals that help them to flocculate such as extracellular polymeric substances (EPS) and the transparent extracellular particulate (TEP) which are larger in size and structure than EPS (Passow, et al. 2001; Manheim and Nelson, 2013). A TEP-like substance has been identified in suspension and biofilms present in WW, saltwater and fresh water resources. TEPs were found in higher concentrations in WW (secondary effluent) than the salt or fresh water (Berman and Holenberg, 2005; Manheim and Nelson, 2013)

Microalgal settling is affected by the growth stage of microalgal cells. It has been reported in a settling study for two microalgal species *Scenedesmus sp.* and *Chlorella vulgaris* that *Scenedesmus sp.* settling greatly improves in the stationary phase. On the other hand, *Chlorella vulgaris* settling was better in the exponential phase (Manheim and Nelson, 2013). In a study for the sinking rates for a four microalgae species (*Asterionella formosa*, *Melosira agassizii*, *Cyclotella meneghiniana*, and *Scenedesmus quadricauda*), sinking rate in the stationary phase was four times faster than that in the exponential phase (Titman and Kilham, 1976). An explanation has been suggested for why small cells like *Chlorella vulgaris* settle better in their exponential phase, related to the culture density: in the exponential phase they have a lower cell density and therefore more total surface area is available for flocculation

4.4.4 Further areas of investigations are required

In this chapter the performance of the different microalgal isolates for nutrients removal from municipal WW secondary effluent was assessed for a batch culture conditions in the following conditions: temperature 22 °C, light intensity 130-150 μmol·photon·m^{-2}·s^{-1}, L:D cycle in hours 16:8, with 2.5% CO_2-enriched air supplementation and mixing at 350 rpm for 10 days. The growth rate, nitrogen, and phosphate specific removal rate and the settleability were used to assess the performance of the microalgae isolates from two contrasting environments (UK and Jordan) in comparison with a reference strain. The algal settleability was our main focus in addition to the rest of the criteria for microalgal candidate selection to reduce the harvesting cost.

However, the successful implementation of an economically viable process using microalgae for WW treatment in open ponds must consider are many additional biotic and abiotic factors that affect the process and algal growth in WW as mentioned in Chapter 1 section 1.1.3. Seasonal variation in abiotic factors affects the microalgal growth and performance. In summer, days are relatively long and the temperature high, whilst winter has short days and low temperatures including frost. Thus, performance of individual algal species and isolates in WW treatment could vary between the seasons and result in fluctuations in the dominant species (Canovas et al.1996, Sutherland et al. 2014a). Therefore, it is advisable to evaluate the impact of seasonal changes to microalgae isolates especially where their selection for integration into a WW treatment system is based predominantly on a performance attribute such as settleability (Av_10, Av_12 and So_3 from the UK and Jordan isolates Jo_2, 12, 18, 29 and 34). This is important because satisfactory performance of the system i.e. low-cost harvesting, is likely to require the maintenance of a monoculture of the selected algal strain. Evaluation of season performance should include assessment of the growth, biomass production, nitrogen removal, phosphorus removal and settleability at the four different seasons (Figure 4.9), but should also take account with seasonal variation in WW characteristics and predators. Such a process could identify a single strain with satisfactory performance across all seasons, but more likely will produce a small collection of strains collectively suited to all seasons.

The WW characteristics are different between different WW types (Cai, Park and Li, 2013). In this study, we evaluated the microalgae performance in the secondary effluent from the municipal WW. Assessing the obtained microalgal isolates that can be easily settled by gravity and good in nutrients removal, like Av_12, Av_10 and So_3 from the UK isolates or like Jo_18, Jo_29 and Jo_34 for their ability to remove nutrients from the industrial or agricultural WW in comparison with a reference strains from the culture collection will extend the application of those microalgae strains for different WW types (Figure 4.9).

Since the microalgal isolates from the UK and Jordan feature common species it would be interesting to study the same species that was isolated from the UK and Jordan in comparison with the same species from the culture collection. Examples include So_3 and Jo_40 and *Chlorella sorokiniana* from culture collection and Av_12 and Jo_34 and *Scenedesmus obliquus* from the culture collection and their performance in growth in WW, nitrogen removal, phosphorus removal, biomass production and composition (Lipid, carbohydrate and protein). This study will determine whether the location of isolation (Jordan and the UK) and the adaptation to the WW (indigenous strains or culture collection strains) affect their performance in treating the WW.

Another investigation needed to manage the seasonal variation in the WWTP conditions, is to start by isolating different microalgae species from the WWTPs and consider the frequency of each obtained species in correlation to the season and in correlation of the location and another factor could be considered as well, like the stage of WW treatment and the type of WW. This can be done by collection different WW samples from different locations and obtained a pure single colony then sequence the obtained isolates for identification and correlate the frequencies of the availability of the species with the season and site of isolation.

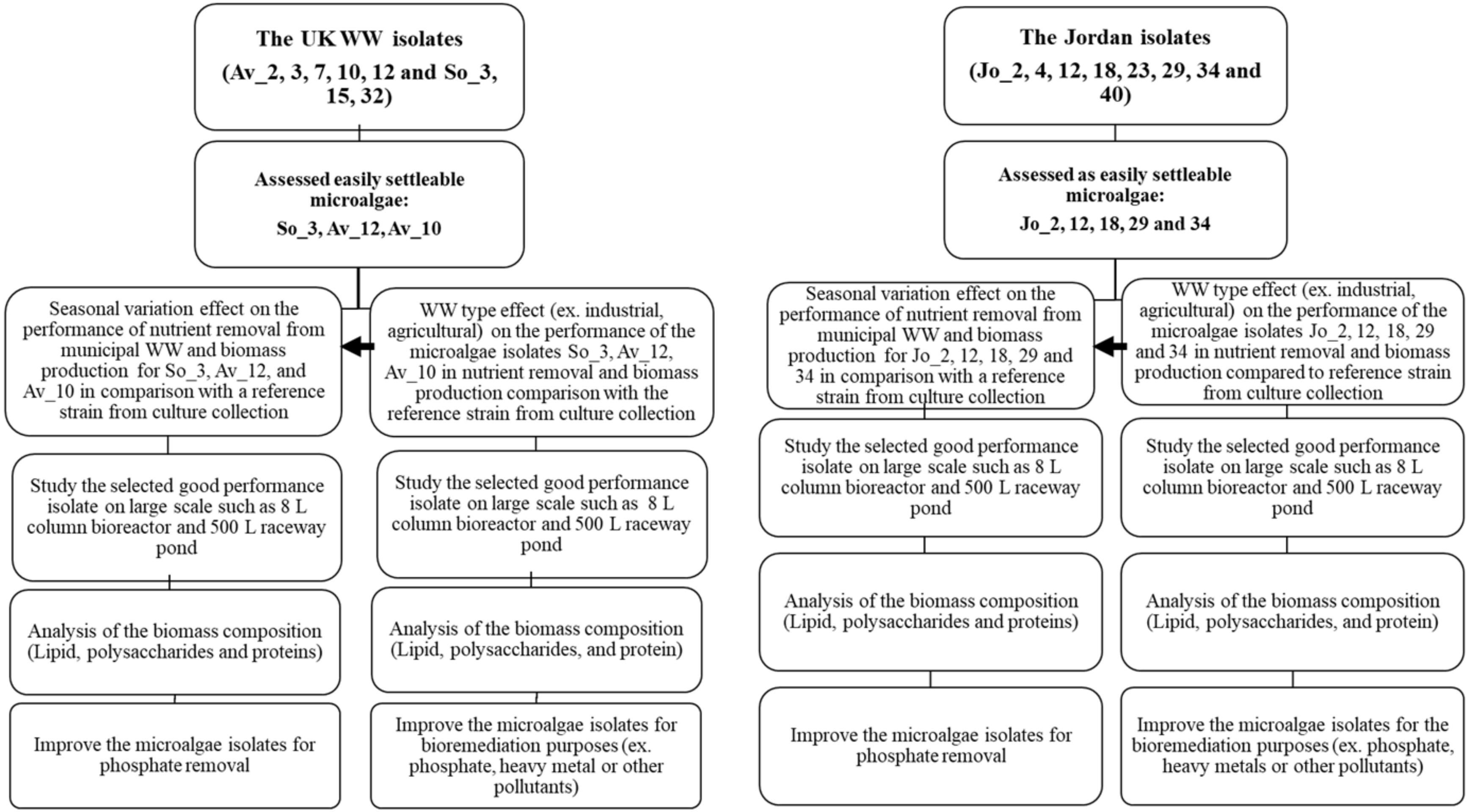

Figure 4.9: Approaches for further investigations for the UK and Jordan isolates to improve the implementation of the microalgae isolates in HRAPs. It is worth mentioning that in a separate part of the project the seasonal evaluation of Av_12 in comparison with the reference strain S. obliquus was done on small scale then the process was scaled up to in 500 L raceway pond

4.5 Conclusion and future work

To get promising candidates from the bioprospected microalgae isolates obtained in chapter 3 for WW treatment and biomass production, it is essential to screen the microalgae isolates from the UK and Jordan for a good performance for growing in real WW, efficiency in removing nitrogen and phosphate, and settleability by gravity, after analysis of the results the samples were ranked and given a score depending on how well they performed in each criterion.

The microalgae isolates were grown in batch cultures for 10 days, daily measurements were obtained for the DW, cell count, nitrate, ammonium, total nitrogen and phosphate. The settleability by gravity was assessed too in a separate experiment. After ranking the microalgae isolates, from the UK isolates the best score was for So_3 followed by Av_12 and Av_10. For upscaling experiments, So_3 was not chosen because it has a problem of sticking on the walls of the container and forms clots under a low mixing velocity, making it hard to grow on a large scale. Regarding the isolates from Jordan, Jo_ 18 and Jo_29 were promising candidates.

During the settling experiment, there was difficulty to determine the settled sludge volume due to unclear phase separation. In the future, evaluating the algal settleability using the percentage of settled biomass (DW) within a time limit rather than measuring the volume, to avoid the phase separation problem is recommended. To seed the algae with the same biomass rather than cell count. Finally, it would be a good idea to assess the recycling effect on algal harvesting as this will help to improve the harvesting process. Assessment of biomass composition of protein, carbohydrate and lipid for downstream applications as a by-product could also help to make the process more economical. A freeze dried microalgal samples has been sent to Bournemouth University, to assess the biomass composition by Fourier transform infrared spectroscopy (FTIR) and Near-infrared (NIR) spectroscopy. In a different part of the WW treatment project using microalgae, Av_12 was chosen for scaling-up it was grown in 8 L column photobioreactors, then in 500 L raceway pond in the University of Bath, those experiments proofed the efficiency of this isolate to treat WW in large scale. currently there is an ongoing trial to grow Av_12 in a pilot scale HRAPs for WW treatment in Beckington WWTP, with total volume of 18000 L treating 1000-3000 L per day.

Chapter 5

The effect PSR1 transcription factor overexpression on the efficiency of phosphate removal and growth of microalgae strains

5.1 Introduction

5.1.1 The need to improve phosphorus removal from wastewater

High concentrations of nitrogen or phosphorus can cause eutrophication in water bodies (DEFRA, 2012; Cai, Park and Li, 2013). In fresh water resources, a high concentration of phosphorus is the major cause of eutrophication, whilst in saline coastal waters, nitrate is a major problem (DEFRA, 2012). The total phosphorus in 74 % of the lakes and the orthophosphate in 45 % of the rivers in the UK failed to achieve targets set by the EU water framework directive, and the Environmental Agency has considered the risk of eutrophication and phosphorus as an important water management issue. As mentioned in Chapter 1 eutrophication of rivers and lakes can affect, the environment by decreasing the biodiversity, the social life such as water sports and the economic sector by affecting tourism and waterside property values (EA, 2012a; The Parliamentary Office of Science and Technology, 2014).

In a study on substance flow analysis of phosphorus in the UK food production and consumption system, Cooper and Carliell-Marquet (2013) reported that in 2009 around 55 kt of phosphorus was received into WWTPs. Of this, only 57% was removed as sewage sludge and the remaining *ca.* 43% (23.5 kt) was discharged in the final effluent to the water systems (Cooper and Carliell-Marquet, 2013). The discharged WW from WWTPs contributes 60-80% of the total phosphorus in UK rivers (EA, 2012a; The Parliamentary Office of Science and Technology, 2014).

Thus, it is important to reduce WW phosphorus levels to the legal limits permitted by Urban Wastewater Treatment Directive for the European Union members. For the WWTP that serves a population of over 100,000, the legal limits for nitrogen and phosphate in discharge WW are 10 mg/L and 1 mg/L respectively (Bloch, 2005). These limits are expected to become more stringent and this will add more pressure to the WW treatment sector, to enhance the systems for phosphorus removal. For comparison, in the USA The National Pollutant Discharge Elimination System (NPDES) limits nitrogen to 10 mg/L, and phosphorus to 0.1 mg/L in discharge from domestic WWTPs (National Oceanic and Atmospheric Administration and Environmental Protection Agency (NOAA/EPA) 1988).

Chapters 3 and 4 describe the bioprospecting and evaluation of microalgae for the removal of nutrients from WW in an open pond system. The promising isolate Av_12 was chosen for integration into an open system due to its good performance in growing and nutrient removal from WW, in addition to its ability to settle under gravity enabling inexpensive separation of treated water and biomass.

The promising performance characteristics of Av_12 were confirmed in a continuous culture using 0.5 m^3 capacity raceway pond system in a glasshouse at the University of Bath. Then Av_12 has been applied to a pilot scale HRAPs for WW treatment in Beckington WWTP, The HARPs are mixed by paddlewheels, they have a total area of 60.3 m^2 and capacity of 18 m^3, it can treat between 1-3 m^3/day depending on the environmental conditions. WW in the ponds was maintained shallow around 12 cm depth to allow for light penetration, increasing the WW depths in the HARP needs more mixing and additional energy input. As mention in Chapter 1, HRAP system has a main challenge of a large area requirement.

The use of HRAPs for nutrient removal could be made more attractive by reducing the footprint i.e. increase the rate of N and/or P removal per unit area of algae or per algal cell, since phosphorus removal is an urgent and technically challenging problem for the WW industry as mentioned above. This chapter explores increasing efficiency of phosphate removal by manipulating the phosphorous uptake machinery of algae, namely by over-expressing the Phosphorus Starvation Response 1 gene (*PSR1*). This was first investigated in the model algae *C. reinhardtii* with aim of applying any promising results to Av_12.

5.1.2 Phosphate uptake by microalgae

To enhance the phosphate removal performance of the microalgae, it is important to understand how microalgae uptake their phosphate. The mechanism of phosphate uptake is controlled by transporters in the plasma membrane and kinetics of phosphate uptake is affected by the form and abundance of these phosphate transporters (Dyhrman, 2016, pp.155-183). The phosphate uptake system has been studied in *Scenedesmus quadricauda* in batch cultures by Jansson (1993). Two kinetic components were identified: the high- and low-affinity components. The high-affinity component had a high affinity for low phosphate concentrations, with phosphate initially bound in exchangeable form in the cell before being transformed to a nonexchangeable form by the low affinity system (Jansson, 1993). The phosphate transportation system in *C. reinhardtii* also includes low-affinity and high-affinity components. The K_m for the low-affinity component is ~10 µM, and 0.1–0.3 µM for the high-affinity component (Shimogawara et al. 1999). The low-affinity component dominates when phosphate is abundant, and is responsible for approximately 80% of total phosphate uptake. After phosphate starvation for 24-hours, the high-affinity system was apparently responsible for all phosphate uptake (Shimogawara et al. 1999; Grossman and Aksoy, 2015, pp.337-374). Jansson (1993) suggested that at low phosphate concentration, the high-affinity system makes the algae competitive with bacteria. If the high-affinity system was constitutively expressed in microalgae not only in low phosphate conditions, would this enhance the phosphate uptake by microalgae and make them more competitive with other organisms?

In *C. reinhardtii* some phosphate transporters have been identified. For the low-affinity phosphate uptake system, one phosphate transporter has been identified: low-affinity phosphate transporter in *C. reinhardtii* (PTC1). PTC1 is similar to the low-affinity transporters Pho87p and Pho90p in the yeast *Saccharomyces cerevisiae* (Grossman and Aksoy, 2015, pp.337-374). The precise function(s) is not fully understood, although the PTC1 transporter in *C. reinhardtii* could have a sensor function (Grossman and Aksoy, 2015, pp.337-374). Two types of high-affinity transporters have been identified in *C. reinhardtii* PTA and PTB. There are four PTA type transporters (PTA1–4), which are similar to the plant-like H^+/P_i co-transporter and the yeast *Saccharomyces* Pho84p high-affinity transporter. *C. reinhardtii* has at least ten PTB-type phosphate transporters (PTB1–9 and PTB12), that are similar to the animal-like Na^+/P_i symporter and the Pho89p high-affinity transporter in *S. cerevisiae* (Grossman and Aksoy, 2015, pp.337-374; Moseley, Chang and Grossman, 2006). Phosphate uptake in cyanobacteria is also controlled by phosphate transporters in a low- and high-affinity system (Dyhrman, 2016, pp.155-183). Manipulating that availability and the density of the abovementioned phosphate transporters may contribute to enhance the phosphate removal from the WW.

Biotic and abiotic factors affect the availability of nutrients and resultant growth of microorganisms. Consequently, microorganisms have evolved a process of acclimatisation. Under low phosphate conditions, Jansson (1993) found that *Scenedesmus quadricauda* expressed a high-affinity phosphate transporter system, to compete with *Pseudomonas* which also has high-affinity transporters. Depending on the character and abundance of transporters they could promote competitiveness against other organisms for nutrients (Grossman and Aksoy, 2015, pp.337-374)

5.1.3 Phosphorus Starvation Response 1 (*PSR1*) in *Chlamydomonas reinhardtii*

Phosphorus Starvation Response 1 (PSR1) is a transcription factor (TF) member of the MYB coiled-coil domain (MYB-CC) family (Wykoff et al. 1999; Moseley, Chang and Grossman, 2006). PSR1 was first described as a component of the phosphate starvation response pathway in the UV light-induced mutant *psr1-1*. This mutant, derived from the CC-125 wild type (WT) strain, has an abnormal response to the environmental level of phosphate (Shimogawara et al. 1999). The *psr1-1* mutant (mt) was further characterised and named *psr1* by Wykoff et al. (1999). This strain was deposited in the *Chlamydomonas* resources centre as CC-4267 psr1-1 mt- referred as (CC-4267 psr1-1) in this study. The PSR1 transcription factor regulates activities for scavenging phosphate from the environment, especially in periods of phosphorus scarcity. PSR1 could also help avoid photo-damage triggered by the over-excitation of photosynthetic electrons at high light intensities (Shimogawara et al. 1999; Wykoff et al. 1999; Moseley, Chang and Grossman, 2006).

In 2006 Moseley and his colleagues studied transcript abundance in *C. reinhardtii* strains WT (CC-125) and the mt (CC-4267 psr1-1) using cDNA microarrays and qRT-PCR to analyse the effect of phosphorus deprivation. In response to phosphorus deprivation, a set of genes was found to increase expression after 24 hours in the WT strain, but their expression remained the same or declined in the *psr1* mt strain. This behaviour is consistent with these genes being regulated by the PSR1 transcription factor. A subset of the genes that are involved in scavenging phosphate from the environment includes: calcium-dependent alkaline phosphatase (*PHOX*); genes that encode putative high-affinity Na^+/P_i symporters (*PTB2*, *PTB3*, *PTB4*, and *PTB5*); and the high-affinity H^+/P_i co-transporters *PTA2* and *PTA3* (Moseley, Chang and Grossman, 2006).

Another group of gene transcripts was found to increase in a PSR1 dependent manner. These potentially encode electron valves, enzymes that function to protect the cell from the damage caused by overexcitation from the electron transport chain of photosynthesis and respiration. Examples include: *PTOX1*, a terminal oxidase present in the plastid; *AOX1*, an isoform of a mitochondrial alternative oxidase; *HYD2*, an iron hydrogenase that can serve as an alternative electron acceptor for PS I; *GAP1*, glyceraldehyde-3-phosphate dehydrogenase (GAP; a Calvin cycle enzyme) a plastid targeted gene; and *GPLV*, the starch phosphorylase gene.

PSR1 has been suggested to play a role in the interaction between regulators of the sulfur and phosphorus starvation response pathways in *C. reinhardtii* (Moseley et al. 2009). Furthermore, PSR1 has been reported to play a key role in triggering cytosolic lipid accumulation (Ngan et al. 2015); the authors concluded PSR1 was involved in transcriptional regulation of lipid metabolism in *C. reinhardtii*, and that PSR1 triggers a moderate accumulation of triacylglycerol (TAG) when there is no stress response. By overexpressing *PSR1* in *C. reinhardtii*, a different lipid profile from the WT strain grown in TAP was noticed. The overexpressing lines accumulated more TAG than the WT strain 4a+, with a major increase in the C18:0 fatty acid component (Ngan et al. 2015).

Recently, the phosphate starvation response has been studied in the *C. reinhardtii* strain CC-125 and CC-4267 psr1-1 by performing transcriptome analysis, qRT-PCR and overexpression of *PSR1* in the *cw15 arg7-8* strain (Bajhaiya et al. 2016). Transcriptome analysis identified transcripts that were upregulated by phosphorus starvation in the WT but misregulated in *psr1* mt. Among these transcripts are a group of genes that encode phosphate homeostasis, such as *PSR1*, *PHOX*, *PTB2*, *PTB4* that were reported earlier to be upregulated (Moseley, Chang and Grossman, 2006). For lipid metabolism, some transcripts were confirmed to be induced in the WT but not in the *psr1* mt under phosphorus starvation conditions including two glycerol 3-phosphate dehydrogenase (*GPD3* and *GPD4*), phosphatidic acid phosphatase (*PAP2*), the dihydrolipoamide acetyltransferase (*DLA*), and one *DGAT* diacylglycerol acyltransferase type 2 (*DGTT2*). Two transcripts of UDP-sulfoquinovose synthase (*SQD1* and *SQD3*) are required for the synthesis of the sulfolipid sulfoquinovosyldiacylglycerol (SQDG), and glycerophosphoryl diester phosphodiesterase *GDP1*. Furthermore, the putative esterase/ lipase transcript g7248 was upregulated by phosphorus starvation. Eight transcripts for the metabolism of starch were upregulated in the WT but not in the *psr1* mt under phosphorus limited conditions. These genes encode two starch phosphorylases (*SP1* and *SP2*), an isoamylase (*ISA3*), two soluble starch synthases (*SSS1* and *SSS5*), and three α-amylases (*AMA2*, *AMA3*, and *AMA-like*1) were reported by Bajhaiya et al. (2016).

The regulation of another subset of electron transport/redox-related and photosynthesis genes has also been confirmed by PCR as altered under phosphorus starvation in the *psr1* mt. Specifically, a predicted cupredoxin (*CSP1*) and ferredoxin isoform (*FDX2*) were highly upregulated by phosphorus starvation in the WT but not in *psr1*. In contrast, the light-harvesting complex II transcript (*LHCBM9*) was highly upregulated by phosphate starvation conditions in the *psr1* mt but not in the WT (Bajhaiya et al. 2016). In the same study, the *PSR1* overexpression lines demonstrated a higher content of starch and number of granules per cell when compared with the WT. This increase in starch content was also correlated with an increase in the expression of some starch metabolism genes. Unlike the results obtained by Ngan et al. (2015) the overexpression lines exhibit a reduction in neutral lipid content (Bajhaiya et al. 2016). The other finding by Bajhaiya et al. (2016) was that the phosphorus content within the overexpression lines, after 3 days of growth in low and high phosphate TAP was higher than that of the WT and the mt.

In low phosphorus conditions, PSR1 triggers the expression of phosphorus scavenging genes such as *PHOX* the calcium dependent alkaline phosphatase for using organic phosphorus and the high-affinity sodium symporters phosphate transporters *PTB2*, *PTB4*. If the PSR1 TF has been constitutively overexpressed in microalgae such as *C. reinhardtii* or Av_12, this may cause constitutive expression of the phosphorus scavenging genes and may enhance phosphate uptake and removal from the WW by the microalgae.

PSR1 has been studied in *C. reinhardtii* but it has been identified in few microalgae that their genome has been sequenced, for Av_12 which is *S. obliquus* it was not identified yet. As mention earlier microalgae are a very diverse group of organisms and this may cause a high variation in *PSR1* gene sequence among the different microalgae species. Which means the PSR1 in *C. reinhardtii* may or may not work if it was transformed into Av_12. The *PSR1* gene encodes a protein located in the nucleus with an MYB-like DNA binding domain and a protein-protein coiled-coil interaction domain working as a transcription factor (TF) (Moseley, Chang and Grossman, 2006). In a study analysing the genome of *C. reinhardtii*, 147 genes were identified as TFs and 87 genes identified as transcription regulators (TRs), which help in regulating gene expression by interacting with TFs, or remodeling the chromatin or other mechanisms (Riaño-Pachón et al. 2008).

The family of MYB TFs is named after the conserved MYB domain, first identified in the *v-Myb* oncogene of an *Avian Myeloblastosis Virus* (*AMV*) (Klempnauer, Gonda and

Bishop, 1982; Li, Ng and Fan, 2015). MYB TFs have been identified in animals, fungi, slime molds and plants and are probably present in all eukaryotes. The MYB family of TFs is one of the largest TF families in plants (Riechmann et al. 2000). In plants this family has a highly conserved MYB DNA-binding domain that has 1-4 imperfect repeats (R) (Li, Ng and Fan, 2015). Each repeat has around 52 amino acid residues that form a helix-turn-helix structure, which has 3 regularly spaced tryptophan amino acids located in the hydrophobic core and mediate the recognition of specific sequences in the DNA (Ogata et al. 1996; Jia et al. 2004; Li, Ng and Fan, 2015). The MYB family has been divided into four subfamilies depending on the position and the number of repeats. The four subfamilies are 4R-MYB, R1R2R3-MYB, R2R3-MYB and 1R-MYB (Dubos et al. 2010; Li, Ng and Fan, 2015). The last subfamily is IR-MYB, to which the PSR1 belongs. To manipulate PSR1 in Av_12 in which the sequence of this gene is not available. It is important to know how much *PSR1* gene is conserved among the different microalgae groups, to give us an idea if it would be possible to design primers to amplify this gene and if the functional domain of this gene is conserved or not among the different species that belong to different families such as *C. reinhardtii* and Av_12.

5.2 Aim and objectives

Enhancement of phosphate removal from WW to make the WW treatment process more efficient, to reduce the size footprint is our main interest. The TF Phosphorus Starvation Response 1 (*PSR1*) in *C. reinhardtii* is critical for regulating the acclimation response to phosphorus starvation (Wykoff et al. 1999). Bajhaiya et al. (2016) further demonstrated that overexpression of *PSR1* in two strains CC-4267 psr1-1, a *psr1* mt and cw15 (cw15 arg7-8) a cell wall-less strain, dramatically increased phosphorous content in cells. This suggests *PSR1* overexpression is a possible strategy to increase the rate of phosphate removal from WW by algae such as Av_12 and thereby reduce the overall size (footprint) of open pond units.

In contrast to *C. reinhardtii*, there is not much information available about Av_12 (*S. obliquus*), the genome is not sequenced yet and the *PSR1* gene is not identified and characterized in this strain. To our knowledge there is only one case in the literature in which *S. obliquus* was successfully transformed (Guo et al. 2013). Another member of the same genus *Scenedesmus almeriensis* was successfully transformed by *Agrobacterium* mediated transformation (Dautor et al. 2014). Since PSR1 has been proven to control part of the phosphate scavenging system in *C. reinhardtii*, the aim of this chapter is to establish a protocol for enhancing phosphate removal by *PSR1* overexpression in *C. reinhardtii* in parallel with same attempts to be applied for Av_12, to overexpress *PSR1* in Av_12 using *PSR1* sequence obtained from *C. reinhardtii*.

The specific objectives of this chapter are:

1. Amplify and sequence the whole *PSR1* gene from the WT *C. reinhardtii* CC-1010
2. Test the sensitivity of *C. reinhardtii* strains (CC-1010 and CC-4267 psr1-1) and Av_12 to the selection marker
3. Optimise the transformation protocol by electroporation for CC-1010 *C. reinhardtii* and Av_12 (*S. obliquus*).
4. Study the effect of *PSR1* overexpression on the algal phenotypes such as growth rate, phosphate removal rate and total phosphorus content in cells.

5.3 Results

5.3.1 Whole *PSR1* gene amplification and sequencing

To enhance the phosphate removal in *C. reinhardtii* and Av_12 by the attempts to overexpression *PSR1* in both species. The whole *PSR1* gene was amplified from *C. reinhardtii* and sequenced to confirm the variation in *PSR1* sequence between the WT strain CC-1010 and the *psr1* mt strain CC-4267 psr1-1. Both the CC-1010 and CC-4267 psr1-1 were purchased from the Chlamydomonas resources centre (section 2.1.4). CC-4267 psr1-1 shows the phenotypic effect of PSR1 absence, such as inability to secrete extracellular phosphatase, and to development a high-affinity affinity phosphate uptake system (Wykoff et al. 1999; Shimogawara et al. 1999). During this chapter, it was used as a control and used for the *psr1* mt complementation test.

The first step in the experimental design was to confirm that CC-4267 psr1-1 contained the appropriate mutation in *psr1* gene i.e. point mutation (deletion) of cytosine at position 458, exon 1, amino acid 153 caused a frame-shift and stop codon in exon 2 (Ngan et al. 2015) and CC-1010 had a WT *PSR1* gene. The whole *PSR1* gene (3 kb) was amplified from CC-1010 and CC-4267 psr1-1 and they were sent for sequencing. The aligment for *PSR1* gene sequences of CC-1010 and CC-4267 psr1-1 were algin beside CC-125 strain (Figure 5.1 A), CC-125 is the parent of CC-4267 psr1-1 that has WT *PSR1* gene (section 2.1.4). As expected CC-4267 psr1-1 contains cytosine deletion at position 458, exon 1, amino acid 153.

Unfortunately, there is no PSR1 homologous protein in *S. obliquus* (Av_12) or any member of the Scenedesmaceae family available in BLAST database. The sequence of *PSR1* from CC-1010 was subjected for nucleotide BLAST search to see if there is a conserved sequence among the different microalgae species that would help for primer design to amplify the *PSR1* homologous gene from Av_12 (Figure 5.1 C). Figure 5.1 B show a phylogenetic tree for the available sequences of *PSR1* homologous genes for microalgae and the monocotyledon maize (*Zea mays*) and the dicotyledon spider flower plant (*Cleome hassleriana*) plants. The PSR1 homologous protein in plants showed a conserved MYB DNA-binding domain with microalgae and this has been reported earlier by Ngan et al. (2015). Figure 5.1. C shows part of the BLAST alignment results for the PSR1 homologous TF sequences that showed the relatively conserved MYB DNA binding domain among the green microalgae and the plant. However, this conserved area was not enough for designing primers, still a question to be answered if this conserved region among the green microalgae is enough for PSR1 to function as a normal TF in *Scenedesmus obliquus* (Av_12) as it is in *Chlamydomonas reinhardtii*?

The difference in the behaviour of the CC-125 and CC-4267 psr1-1 to phosphorous starvation was reported, CC-125 divided 3 to 4 times after transfer into TA medium (phosphorus free medium, phosphate was replaced with 1.5 mM KCl), whilst the mt strain CC-4267 psr1-1 divided only once (Wykoff et al. 1999). As a further check of the strains obtained from the Chlamydomonas resource centre, CC-1010 and CC-4267 psr1-1 were grown in a phosphate depleted TA medium (Figure 5.2) and monitored for the appropriate behavior. Following an initial period of 48h where cell density declined sharply and bleached then recovered. Whereas, the cell density in the mt decreased immediately and continously.

The sequence analysis of the *PSR1* gene in CC-1010 and CC-4267 psr1-1 strains combined with the gowth characteristics of both in phosphorous depleted media suggested that the correct strains were obtained from the resource centre.

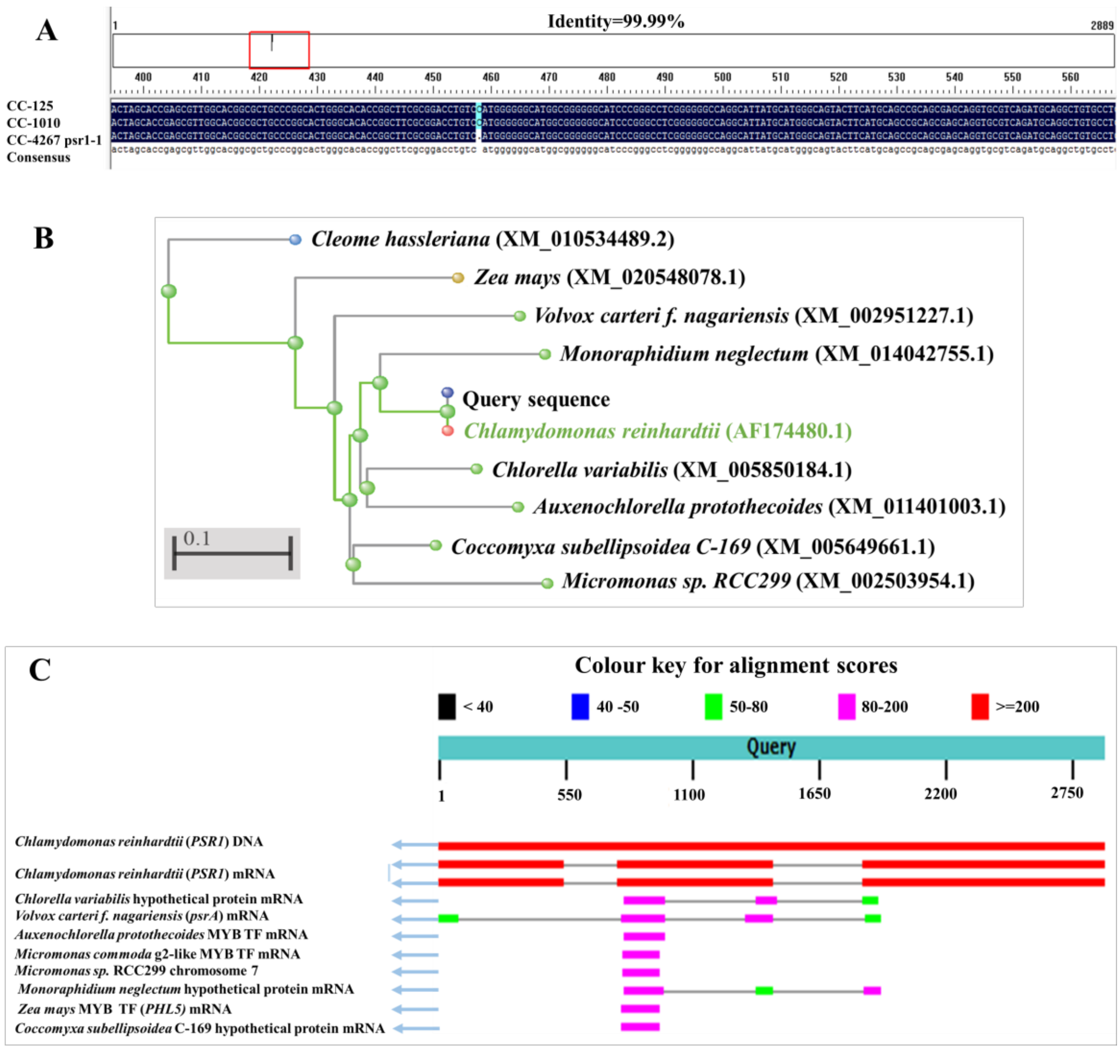

Figure 5.1: Amplification and characterisation of *PSR1* gene sequences. *(***A***)* Single point mutation was detected in the mt strain, cytosine deletion at position 458. **(B)** Neighbour joining tree for the available *PSR1* sequences in BLAST for the microalgae, maize, and the spider flower. This tree has been produced using BLAST pair-wise alignment, unknown is *Chlamydomonas reinhardtii PSR1* gene from CC-1010 strain. **(C)** The graphic is an overview of the sequences aligned to the *PSR1* DNA sequence of *Chlamydomonas reinhardtii* CC-1010. The horizontal bars are colour coded by score and viewing the level of the alignment on the query sequence.

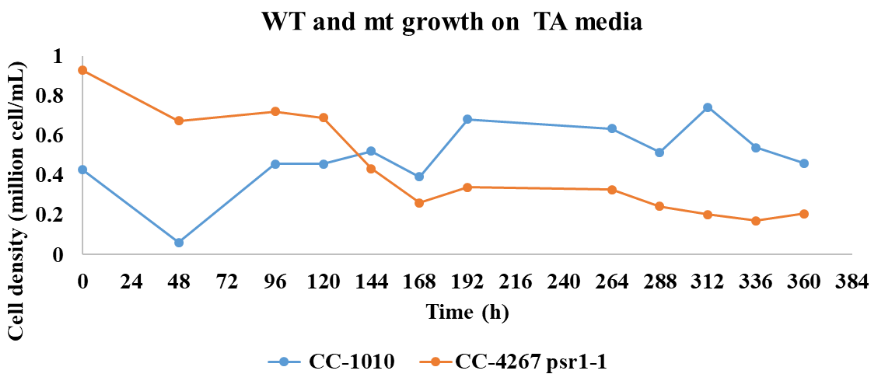

Figure 5.2: Characterisation of CC-1010 and the CC-4267 psr1-1 growth in phosphate depleted TA medium. The strains were grown at 50 µmol·photon·m^{-2}·s^{-1}, L:D 16:8 cycle in hours and 25 °C.

5.3.2 Antibiotic sensitivity for *C. reinhardtii* and Av_12

To optimize the protocol for transforming *C. reinhardtii* and Av_12 with the *PSR1* expression construct, a proper selection marker is essential to select the transformed colonies with the expression construct. pOpt_mVenus_Paro vector has the *aphVIII* gene that confers resistance for paromomycin antibiotic as a selection marker. Since different microalgae are different in their response to the same antibiotics (Díaz-Santos et al. 2013). Antibiotic sensitivity test to the paromomycin antibiotic was carried on to determine the appropriate concentration of the antibiotic for *C. reinhardtii* strains and Av_12

A spot test for the paromomycin sensitivity of *C. reinhardtii* (CC-1010, CC-125 and CC-4267 psr1-1) not previously reported was tested (Figure 5.3 A). The concentration of paromomycin commonly used *Chlamydomonas* transformation is 10 mg/L (Lauersen, Kruse and Mussgnug, 2015). The three *Chlamydomonas* strains did not grow on the paromomycin/TAP plates even when the concentration was reduced 5 mg/L. In contrast, Av_12 proved much more resistant to the antibiotic with growth observed at paromomycin concentrations of between 5 and 10 mg/L. This suggests that 100 mg/L would be effective in the selection process (Figure 5.3 A).

Growth monitoring for CC-1010 and Av_12, when grown in liquid cultures showed that *Chlamydomonas* CC-1010 is sensitive to paromomycin, the percentage of the change in cell density (death) from day 0 to day 7 was -100 % reduction in cell density at 10 mg/L and 100 mg/L paromomycin concentration (Figure 5.3 C), whereas, the change in the percentage of the cell density for Av_12 was −8% at 10 mg/L and −99 % reduction at 100 mg/L. These results support the use of 10 mg/L paromomycin for *C. reinhardtii* and

100 mg/L paromomycin for Av_12. However, during the optimisation of the transformation experiment there was growth in the Av_12 negative control plates of paromomycin at 100 and 200 mg/L suggesting that at those concentrations Av_12 still can survive. There was no growth in the negative control plates containing 300 mg/L of paromomycin. Accordingly, 10 mg/L of paromomycin was used for *Chlamydomonas* strains, and 300 mg/L was applied to Av_12.

5.3.3 Assembling of pOpt_mVenus_Paro_PSR1 construct.

To achieve a strong constative expression for *PSR1* gene in the microalgae strain. The *PSR1* needs to be inserted in a vector that has a strong constitutive expression promotor like pOpt_mVenus_Paro vector to be transformed into the microalgal strain. The nuclear expression vector pOpt_mVenus_Paro (Lauersen, Kruse and Mussgnug, 2015) was selected because it has: a strong constitutive *HSP70A-RBCS2-i1* promoters, paromomycin antibiotic as selection marker, The RBCS2 3′ untranslated region (3′UTR) as regulatory element and the protein is tagged with a StrepII affinity tag (*StrepII-TAA*) as reporter (Figure 5.4 A). The gene of interest expression cassette and the antibiotic selection marker cassette are separated but controlled by the same constitutive promoters and the RBCS2 3′ untranslated region (3′UTR) regulatory element. Section 2.2.5 provides more details of pOpt_mVnus_Paro vector.

In order to construct a cassette for *PSR1* overexpression the entire *PSR1* gene (2889 bp) was amplified from the WT strain CC-1010. Restriction sites for *Nde*I and *Eco*RI were added to the gene using PSR1F-RS-1and PSR1R-RS-1 primers (Figure 5.4 B) to facilitate insertion of the gene into the expression vector pOpt_mVenus_Paro (Lauersen, Kruse and Mussgnug, 2015; Figure 5.4 A). The vector was double digested with *Nde*I and *EcoR*I enzymes to remove the mVenus reporter (Figure 5.4 C) and replaced with the *PSR1* gene. After the ligation, the construct produced was transformed into *E. coli* and the construct then checked by colony PCR and double digestion to confirm the presence of the *PSR1* gene (Figure 5.4 D). In the colony PCR, RBC2i-F1 and PSR1R-RS-1 primers were used to amplify the whole gene 3 kb fragment. The *PSR1* gene in the construct directly sequenced for validation. The internal primers used were: RBCS2i-F1, PSR1-IR1, PSR1-IF2, PSR1-IR2, PSR1-IF3, PSR1-IR3, PSR1-IF4 and 3`UTR-R4 (listed in Table 2.8 and figure 2.3 illustrate their position). The construct was then linearized using *Xba*I to produce an approximately 8.6 kb fragment (Figure 5.4 E). Plasmid linearization increases the efficiency of plasmid integration into the nuclear genome of recipient algal cells (Leon and Fernandez, 2007, pp.1-11).

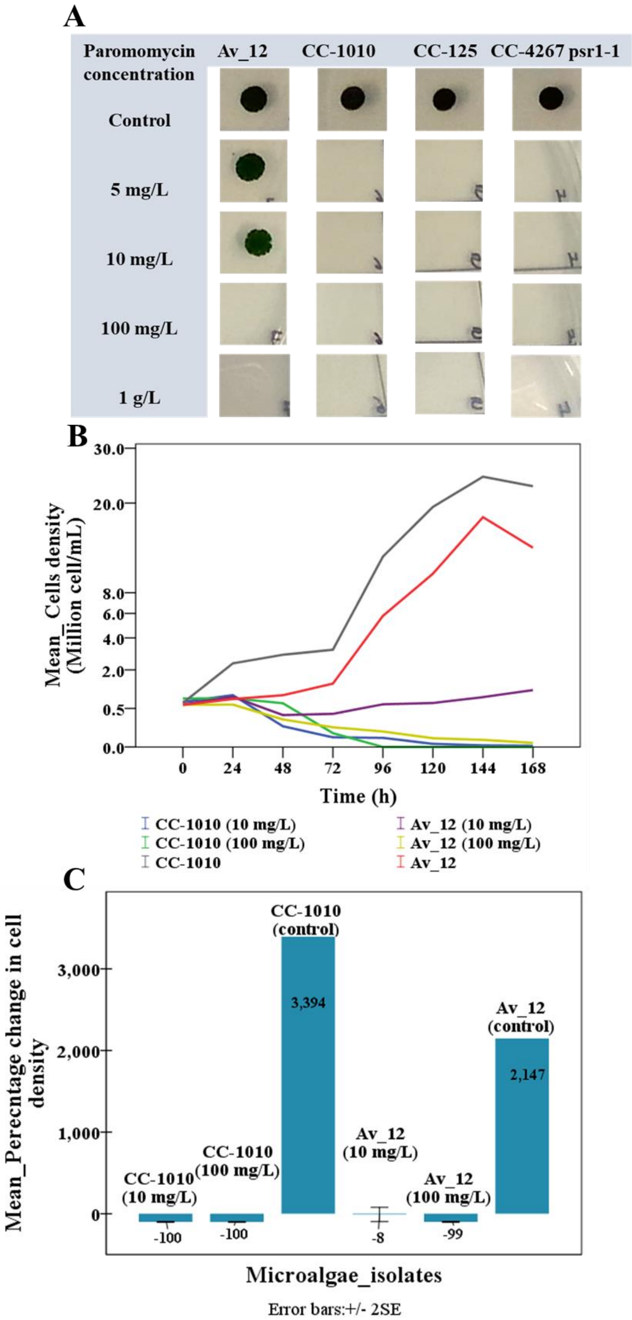

Figure 5.3: Establishing appropriate antibiotic concentration for selection using paromomycin. The growth conditions were 50 µmol·photon·m^{-2}·s^{-1}, L:D 16:8 cycle in hours and 25 °C. **(A)** Spot test on TAP agar plates for CC-1010, CC-125, CC-4267 psr1-1 and Av_12 strains at four different paromomycin concentrations (5, 10, 100 and 1000 mg/L), the control was without antibiotic. **(B)** The cell density for CC-1010 and Av_12 were grown in liquid cultures with different concentration of the antibiotic and without antiobiotic, CC-1010 and Av_12 were grown in triplicats at two concentration of the antibiotic paromomycin (10 mg/L and 100 mg/L); the control treatment was without antibiotic and no replicates so there were no error bars. **(C)** The change in the cell count of each treatment in the liquid cultures from the first to last day of the experiment expressed as a percentage.

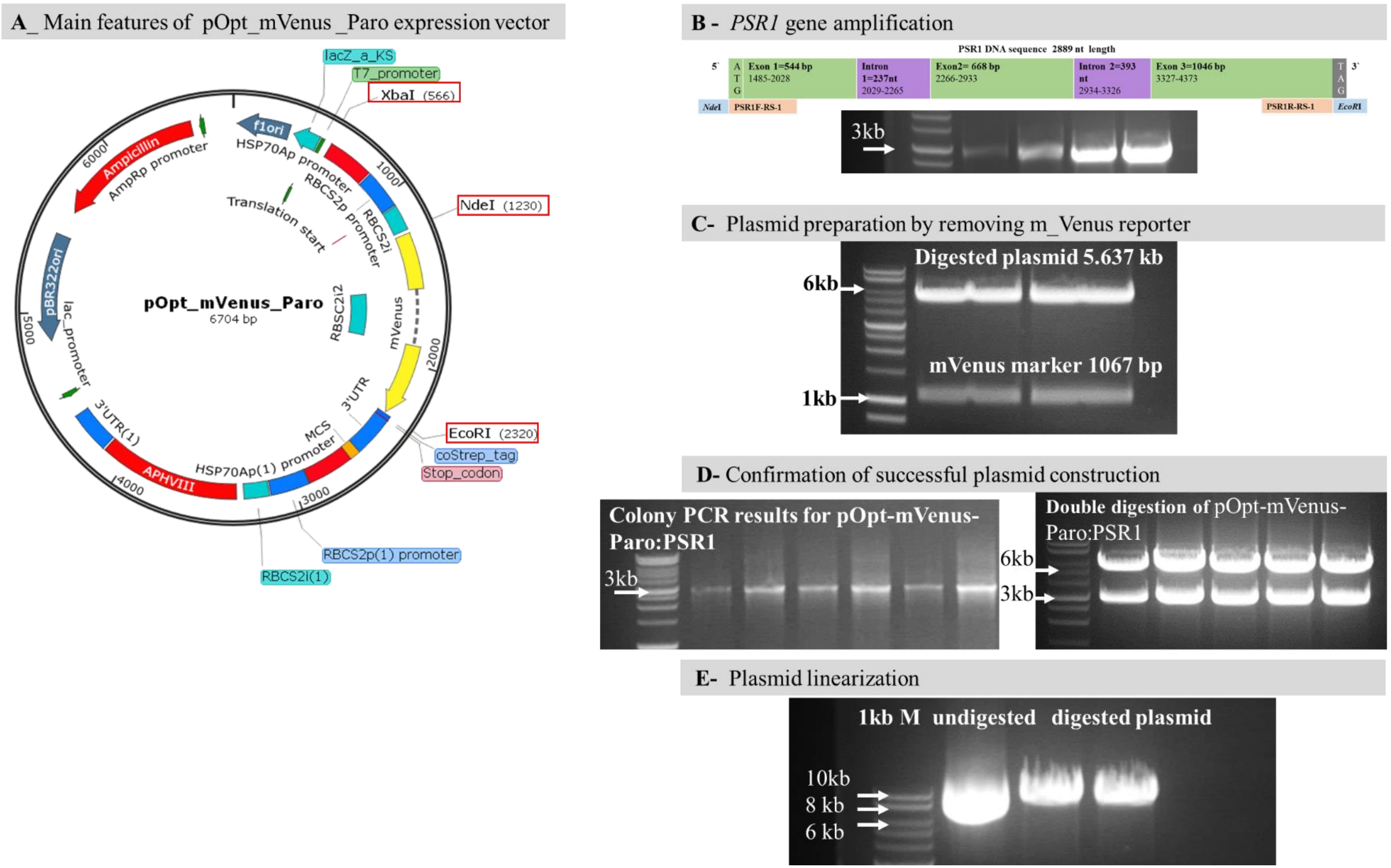

Figure 5.4: Construct assembly. (A) Map of the main features of pOpt_mVenus_Paro expression vector for *Chlamydomonas reinhardtii*. **(B)** The amplification of PSR1 TF from CC-1010 WT strain surrounded with two restriction sites. **(C)** Removing the m_Venus reporter to be replaced by *PSR1* gene. **(D)** Confirmation of successful ligation of the gene into the vector by colony PCR and double digestion of the plasmid to release the *PSR1* fragment. **(E)** Linearisation of the plasmid by digestion with *XbaI*.

5.3.4 Nuclear transformation by electroporation was successful for *C. reinhardtii* strains but not for Av_12

This section presents the attempts to optimize a transformation protocol for microalgal strains by electroporation. The three microalgae Av_12, CC-1010 and CC-4267 psr1-1 were subjected to electroporation trials with different published protocols optimized for *C. reinhardti* and *S. obliquus* with and without modifications.

Transformation of *C. reinhardtii*

Using the GeneArt max efficiency reagent (Invitrogen) protocol on Gene Pulser X Cell electroporator, *Chlamydomonas* strains CC-1010 and CC-4267 psr1-1 were successfully and effectively transformed with pOpt_mVenus_Paro vector with *PSR1* and without *PSR1* gene, For CC-1010 strain that was transformed with pOpt_mVenus_Paro_PSR1, around 600 paromomycin resistant colonies obtained the transformation efficiency was for CC-1010 181 paromomycin resistant colony /µg of plasmid (Figure 5.5). No growth was detected on negative control plates receiving algal cells electroporated without plasmid. The putative transformed colonies appeared on the plates after 5–7 days.

CC-1010 transformed with pOpt_mVenus_Paro only | **CC-1010 transformed with the pOpt_mVenus_Paro_PSR1**

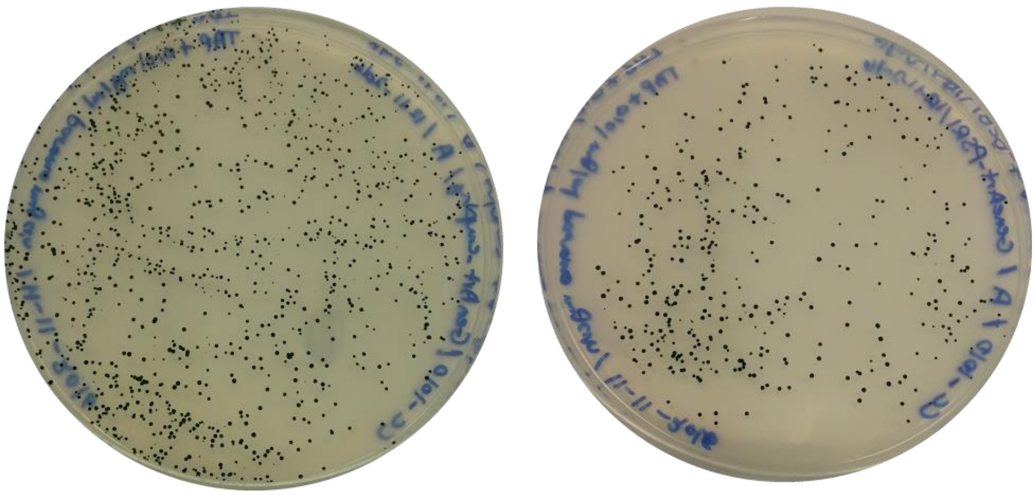

Figure 5.5: Putative transformed CC-1010 colonies growth on TAP plates with 10 mg/L paromomycin. The growth conditions were 50 µmol·photon·m^{-2}·s^{-1}, L:D 16:8 cycle in hours and 25 °C.

Random colonies were picked and subcultured on a TAP medium plate with 10 mg/L for two passes to ensure the stability of paromomycin resistance. Next a 50 mL tube containing 5 mL of TAP medium only without antibiotic was inoculated by a single colony and incubated at the conditions of the growth chamber incubator SANYO (section

2.1.3) with mixing for 7–10 days. DNA was then isolated and subjected to PCR to conformation the presence of the construct using PSR1-OEC-F and PSR1-IR1 (section 2.2.4, Table 2.8) to amplify 1168 bp of the vector and our gene of interest (GOI) (Figure 5.6 A). A paromomycin resistance gene (*aphVIII*) specific primer Paro-R-F and AR-OEC_R primer were used to screen colonies transformed with vector-only, this produced a 1140 bp band (Figure 5.6 B). Six colonies that were positive according to the PCR analysis (CC-1010_A-2, A-6, A-9, B-2, B-18, and B-34) were selected from *C. reinhardtii* CC-1010 strain transformed colonies with the vector and the GOI along with four from the same strain CC-1010 transformed with the vector only (CC-1010_V-2, V-6, V-7, and V-10). For *C. reinhardtii* mt strain CC-4267 psr1-1, ten positive colonies were selected (CC-4267_3, 5, 10, 31, 35, 37, 40, 48, 49 and 50).

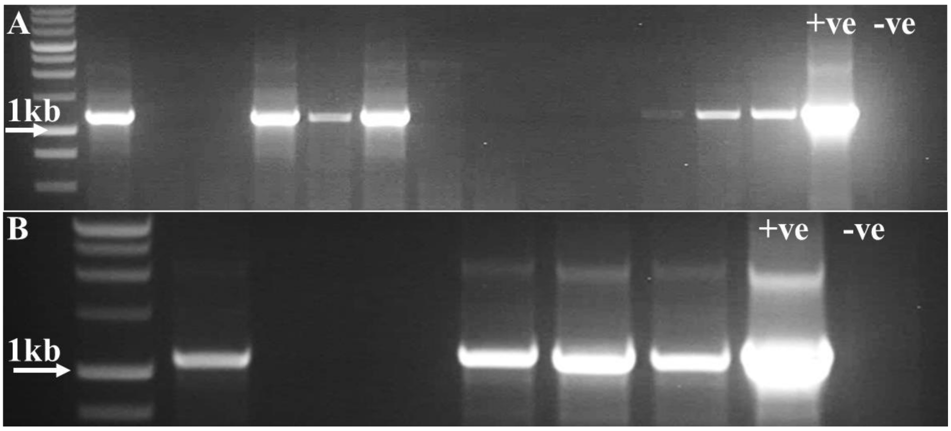

Figure 5.6: Confirming presence of the *PSR1* gene and vector in the selected putative transformed colonies. (A) An approximately 1.2 kb fragment has been amplified from the transformed colonies to confirm the presence of *PSR1* gene and vector. **(B)** Amplified fragment from the antibiotic resistance cassette to confirm the presence of the vector in the transformed colonies with only the vector.

Transformation of Av_12

All the transformation trials by electroporation for Av_12 were unsuccessful. There was no growth in the TAP plate with 300 mg/L paromomycin (no colonies appeared). For all the samples that are transformed with, the pOpt_mVenus_Paro_PSR1 and pOpt_mVenus_Paro as a control. The same result was for the negative control which was electroporated into cells only without vector. A published protocol was established for *S. obliquus* transformed with electroporation (Guo et al. 2013) was followed and modified, and GeneArt max efficiency reagent (Invitrogen) protocol was followed and modified it did not work too. The modification in the electroporation conditions included changing the electroporation buffer (TAP, TAP-40 mM sucrose and osmosis solution), voltage, resistance, DNA concentration and sample size. Also, a square wave multi pulses were applied as mention in (section 2.2.12), in which a multiple pulses were delivered to the cells. The voltage, the number of pulses, the duration and the intervalses between pulses were manipulated in those trials.

5.3.5. The putative overexpression lines grew in low phosphate TAP as fast as the wild type strain

Before evaluating the effect of *PSR1* over expression on algal growth, phosphate removal from environment and phosphorus content in the cell. It was necessary to select a high *PSR1* expressing lines, or at least a range of expression levels. Some factors such as the integration site (position effects) and copy numbers will influence level of *PSR1* expression. Lines with a high expression level is the target, to identify suitable lines within the selected transformants. The hypothesis was under stressful low-phosphate conditions, lines able to overexpress *PSR1* are able to scavenge phosphate more effectively and therefore adapt more quickly (start dividing) achieve higher growth rates than WT cells in phosphate limitation.

Therefore, a simple screening process was conducted for the six CC-1010 putative over expression lines that harbour the pOpt_mVenus_Paro_PSR1 construct (CC-1010_A-2, A-6, A-9, B-2, B-18, and B-34), CC-1010 and CC-4267 psr1-1 which lack the endogenous PSR1 TF was used as negative control. They were grown in Low-P_i-TAP medium with 100X lower phosphate (section 2.1.2). The experiment was performed in 96-well microplates and the OD 750 nm was measured to evaluate the growth of putative overexpression lines for 7 days. The putative transformed microalgae lines (CC-1010_A-2, A-6, A-9, B-2, B-18, and B-34) grew with similar growth rate to CC-1010 strain. However, analysing the specific growth rate using a Kruskal-Wallis test, revealed no significant difference between their specific growth rate in Low-P_i-TAP medium (Figure 5.7 B).

Although there was no significant improve in the growth rate when the putative overexpression lines were grown in Low-P_i-TAP medium, this does not mean that the putative overexpression lines are not overexpressing *PSR1*. This experiment evaluated the specific growth rate rather than the phosphate removal. There is the possibility that the putative overexpression lines take up phosphate faster than the others, but since there is a limited amount of phosphate for all the isolates they cannot grow freely under these limited conditions. Thus, two random putative overexpression lines were chosen CC-1010_A-6 and CC-1010_B-2.

5.3.6 A transformant successfully complemented the extracellular phosphatase production

The *psr1* mt strain CC-4267 psr1-1 was transformed with the *PSR1* overexpression construct, to determine whether the construct complemented the mt effect. The rationale was as follows: since the *psr1* mt cannot produce extracellular phosphatase (Shimogawara et al. 1999; Wykoff et al. 1999) they cannot therefore grow in media where the only source of phosphorous is organic phosphorus. Thus, successful complementation of PSR1, which would lead to the production of extracellular phosphatase, they would be able to uptake phosphate and grow in the same manner of CC-125 strain the WT parent for the strain CC-4267 psr1-1. One line, CC-4267_50, showed complementation of the mutation out of ten lines tested (Figure 5.8). This might be due to the effect of the insertion position, since the GOI integrates into the microalgae genome randomly. That is the GOI can be inserted in a compact structure of the chromatin that prevents the transcription of the GOI (Neupert, Karcher and Bock, 2009).

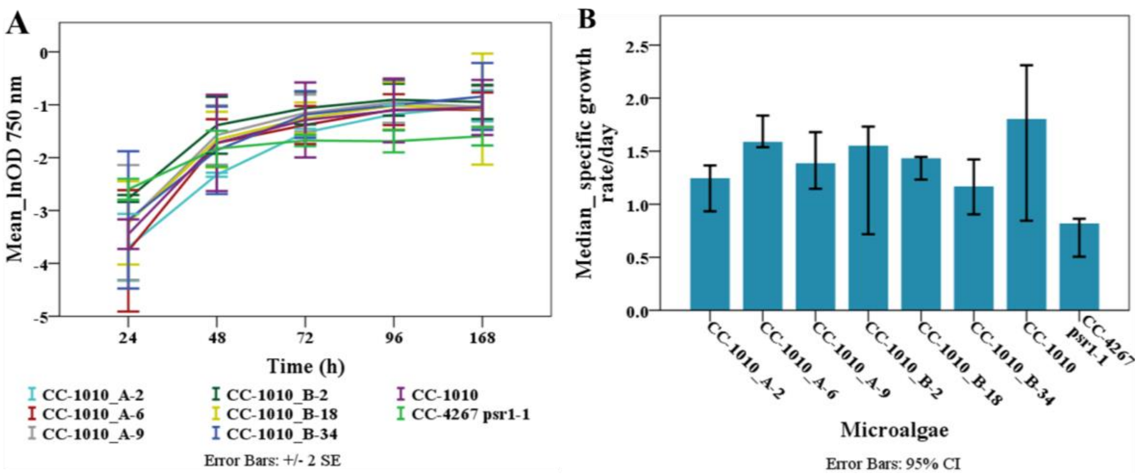

Figure 5.7: There is no significant difference between the growth of the putative overexpression lines and the WT in Low-Pi-TAP medium. (A) The growth curve for all the lines. **(B)** Median of the specific growth rate. Kruskal-Wallis test analysis at significance level 0.05 showed no significant difference between the different samples (error bars represent 95% confidence intervals; *n* = 18). All putative overexpression lines, CC-1010_WT and CC-4267 psr1-1_mt were grown in Low-Pi-TAP TAP (10 µM phosphate) in Sanyo environmental test chamber conditions (50 µmol·photon·m^{-2}·s^{-1}, L:D 16:8 cycle in hours and 25 °C) without shaking.

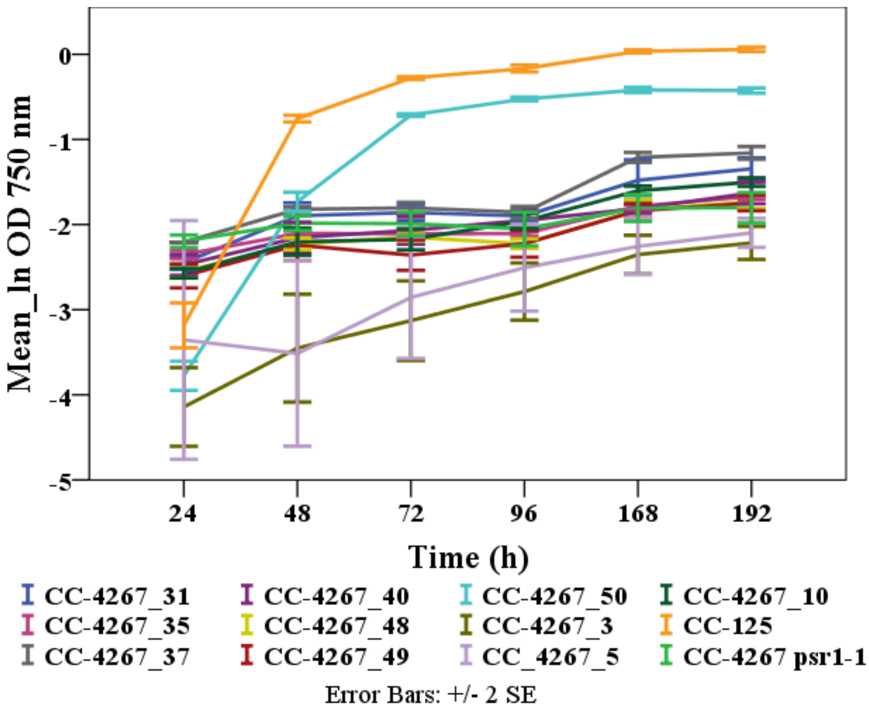

Figure 5.8: Complementation of the *psr1* mt. Out of the ten microalgae lines selected with the *PSR1* overexpression construct, one, CC-4267_50, grew on organic phosphate source at a rate similar to the strain CC-125 (error bars represent standard error; *n* = 6). The samples were grown in TAP medium in which the phosphate was replaced with 100 µM glucose monophosphate. The 96-well microplates were kept in Sanyo environmental test chamber conditions (50 µmol·photon·m^{-2}·s^{-1}, L:D 16:8 cycle in hours and 25 °C) without shaking.

5.3.7 Phenotype characterisation of putative overexpression lines

This experiment was designed to check the impact of *PSR1* overexpression in the putative overexpression lines, on the specific growth rate, phosphate removal and phosphate content in the cells. These effects were studied because PSR1 trigger the expression for a part of phosphorus scavenging system (*PHOX*, *PTB2*, *PTB4*) in the microalgae (section 5.1.3).

The effect of the overexpression was studied in low-P_i-TAP (10 µM PO_4) and normal TAP (1 mM PO_4). Five *Chlamydomonas* cultures were chosen to be grown: three strains as controls CC-4267 psr1-1, CC-1010, CC-1010_Vector; and two putative overexpression lines CC-1010_A-6 and CC-1010_B-2. The microalgae were grown in 500 mL bottles in final volume of 400 mL at cell density ~10^6 cells/mL for 10 days, at light intensity of 70 µmol photon·m^{-2}·s^{-1}, L:D 18:6, 22 °C, and mixed at 250 rpm. During the experiment, a daily measurement was taken for the dry weight, cell count, and OD to assess growth of the microalgae, phosphate in the media and total phosphorus in the algal cells to assess the efficiency of phosphate removal.

The putative overexpression lines grew as rapidly as the wild type in low phosphate

The first phenotype was evaluated for the *PSR1* putative overexpression lines was their specific growth rate in TAP media with low and normal phosphate concentration beside the controls strains. To ensure there was no phosphate transported with the microalgae from the media, they were washed twice with TA medium (section 2.1.2). In general, newly subcultured microalgae go through a lag phase to adapt to the new media and new growth conditions, but the reaction of the transgenic lines, CC-1010_A-6 and CC-1010_B-2, was markedly different to cultures of the control lines, CC-4267 psr1-1, CC-1010, CC-1010_Vector. The transgenic lines initially bleached but then recovered their green colour after about 72 hours (Figure 5.9 A), then continued to grow normally. The algal cells for each strain in the two different media were examined under the microscope a day before the end of the experiment (Figure 5.9 B).

The growth curves of the microalgae in Figure 5.10 A are based on dry weight content. These curves showed that the transgenic lines at Low-P_i-TAP and normal TAP medium did not grow in the first 48 hours, and in fact there was a 19-28% reduction in the biomass among the putative overexpression strains. Growth in biomass was measurable after 72 hours. As shown in Figure 5.10 B, the cell density of the two putative over expression lines decreased at the beginning of the experiment and also started to increase after 72 hours. The specific growth rates of the microalgae were also measured and analysed (Table 5.1). Statistical analysis of the growth rate using a Kruskal-Wallis test showed there was no significant difference in the specific growth rate between the putative over expression lines (CC-1010_A-6 and B-2) in comparison with the WT strain in low-P_i-TAP (indicated by black asterisks, *****; Figure 5.10 C). This result agrees with the those of (Bajhaiya et al. 2016) who found that under low and high phosphate concentrations there was no significant difference in the growth rate of overexpression lines and WT. In the present work, there was a significant difference between the specific growth rate of the WT and the putative overexpression lines in comparison with CC-1010_Vector and the *psr1* mt strain CC-4267 psr1-1 (Figure 5.10 C). In TAP medium, in which phosphate availability was not limited, the putative overexpression line CC-1010_A-6 had the highest specific growth rate that was significantly different from the rest of the strains including the WT (indicated by blue asterisk, *). CC-1010_B-2 grew as fast as the WT and mt in normal TAP when phosphate availability was high (Figure 5.10 C).

To compare the growth of the same microalga when grew in low-P_i-TAP or normal TAP was analysed, the significant difference is indicated by the number sign # (Figure 5.10 C). The specific growth rate of putative *PSR1* overexpression line CC-1010_A-6 and the mt strain increased when the available phosphate increased in the medium. This effect was not observed for CC-1010_B-2, WT and CC-1010_Vector. Limiting phosphate affected the growth of the mt strain since when it was grown in low-P_i-TAP it had the lowest specific growth rate and was significantly different from that of the other strains. This agrees with earlier results reported that the growth of the *psr1* mt was inhibited under low phosphate conditions in comparison with WT (Wykoff et al. 1999; Bajhaiya et al. 2016).

CC-1010_B2 removed phosphate more rapidly than the wild type strain

This section evaluates the effect of *PSR1* overexpression on the efficiency of the putative overexpression microalgae in removing the phosphate from the environment in comparison with the WT, CC-1010_Vector and the mt. A daily measurement of the phosphate level in the medium was conducted in order to assess the phosphate removal capacity of the different strains (Figure 5.11 A). The performance of the different microalgae strains/lines varied in the low-P_i-TAP and in the normal TAP medium.

Phosphate removal started immediately for the strain CC-1010 in the low and normal TAP medium. However, there was a measurable increase in phosphate concentration in both low-P_i-TAP and normal TAP media for the *PSR1* transformed lines CC-1010_A-6 and CC-1010_B-2. This could be due to lysis of dead cells which would release phosphate into the medium. This would be consistent with the 19%-28% reduction in the biomass noted in the previous section. Alternatively, the secretion of phosphorus to the media by cells could have occurred, as indicated by the decrease in biomass phosphate content of between 20%-40% observed after 24 hours. After the *PSR1* transgenic lines recovered from bleaching they removed phosphate from the medium more rapidly than the other strains (Figure 5.11 B). Low-P_i-TAP CC-1010_B-2 had the highest specific phosphate removal rate followed by the CC-1010_A-6, significantly different to the remaining samples including the WT strain. In the normal TAP medium, CC-1010_B-2 produced the best phosphate removal performance with significant difference from the WT strain followed by CC-1010_A-6 which behaved similar to the WT in high phosphate conditions (Figure 5.11 B). A significant difference was observed between the specific phosphate removal for the same isolate in the two different phosphate concentrations. Only CC-1010_B-2 behaved differently between the high and Low-P_i-TAP (Figure 5.11 B). Table 5.1 presents the actual values of the specific phosphate removal rate for the samples.

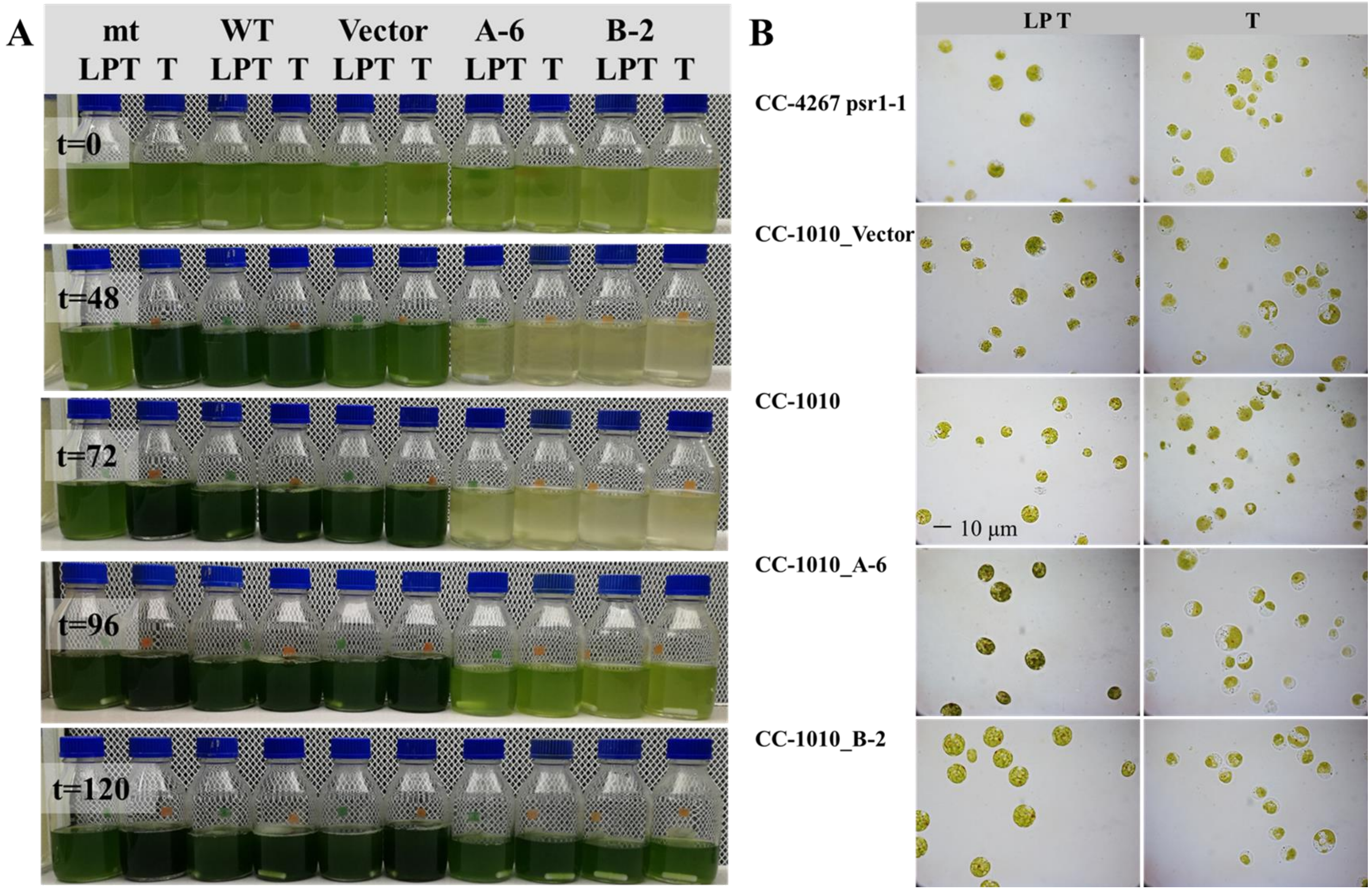

Figure 5.9: Status of the cultures of the microalgae strains that were grown in Low-P$_i$-TAP (LPT) and normal TAP (T) medium. (A) Microalgae cultures growing in 500 mLbottles under the following conditions: 70 µmol photon· $m^{-2}{\cdot}s^{-1}$, L:D 18:6 cycle in hours, 22 °C and mixing at 250 rpm, some of which appear bleached. (**B**) Microalgae cells at the end of the experiment after 192 hours, at 1000 X magnification. Key to strains: mt, CC-4267 psr1-1; WT, CC-1010; Vector, CC-1010_Vector; A-6, CC-1010_A-6 and B-2, CC-1010_B-2.

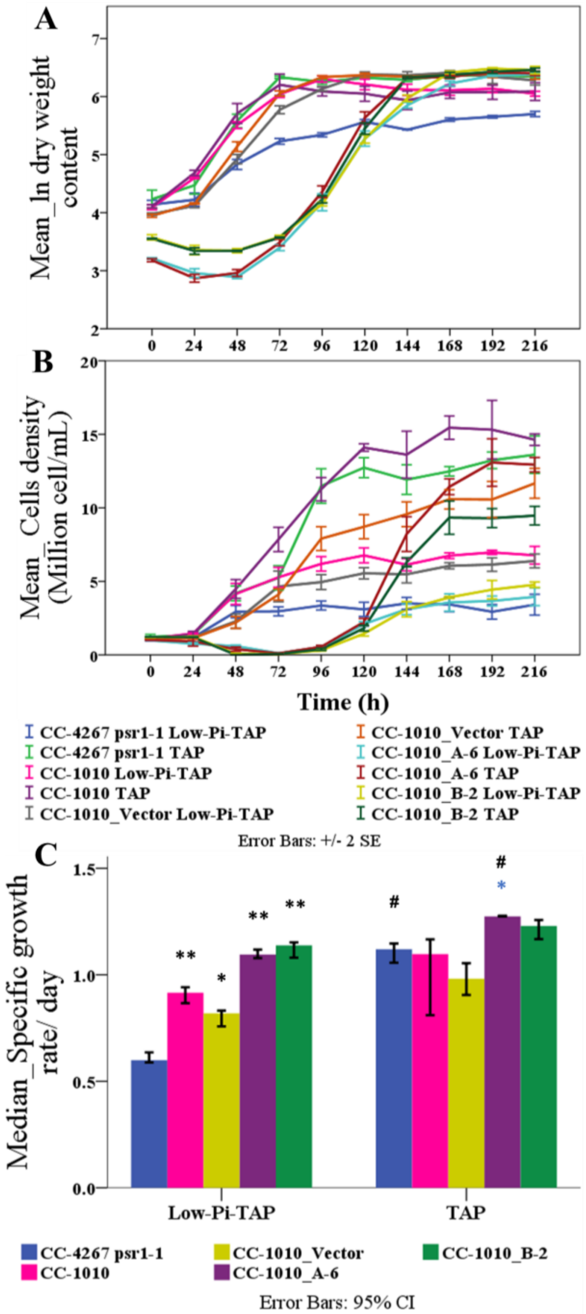

Figure 5.10: The growth of the microalgae under Low-P$_i$-TAP and normal TAP. Microalgae were grown in TAP medium with different phosphate concentrations; low phosphate TAP (Low-P$_i$-TAP) with 10 µM PO_4^{-3} and TAP with 1 mM PO_4^{-3} medium. The growth conditions were 70 µmol photon· $m^{-2} \cdot s^{-1}$, L:D 18:6 cycle in hours, 22 °C and mixing at 250 rpm, **(A)** Growth curves based on dry weight content (error bars represent standard error; *n* = 3). **(B)** Cells density throughout the experiment (error bars represent standard error; *n* = 3). (**C**) Median specific growth rate for the five tested microalgae. Significant differences in the specific growth rate among strains/lines grown in low phosphate TAP medium are indicated by black asterisks (*). A blue asterisk (*) indicates the significant difference in specific growth rate compared to the WT strain in normal TAP medium. Number sign (#) indicates the significant difference between the same strain/line in the two media. Samples were statistically analysed using a Kruskal-Wallis test followed by homogenous subsets analysis based on asymptotic significances at the 0.05 significance level (error bars represent the confidence intervals level at 95%; *n* = 3).

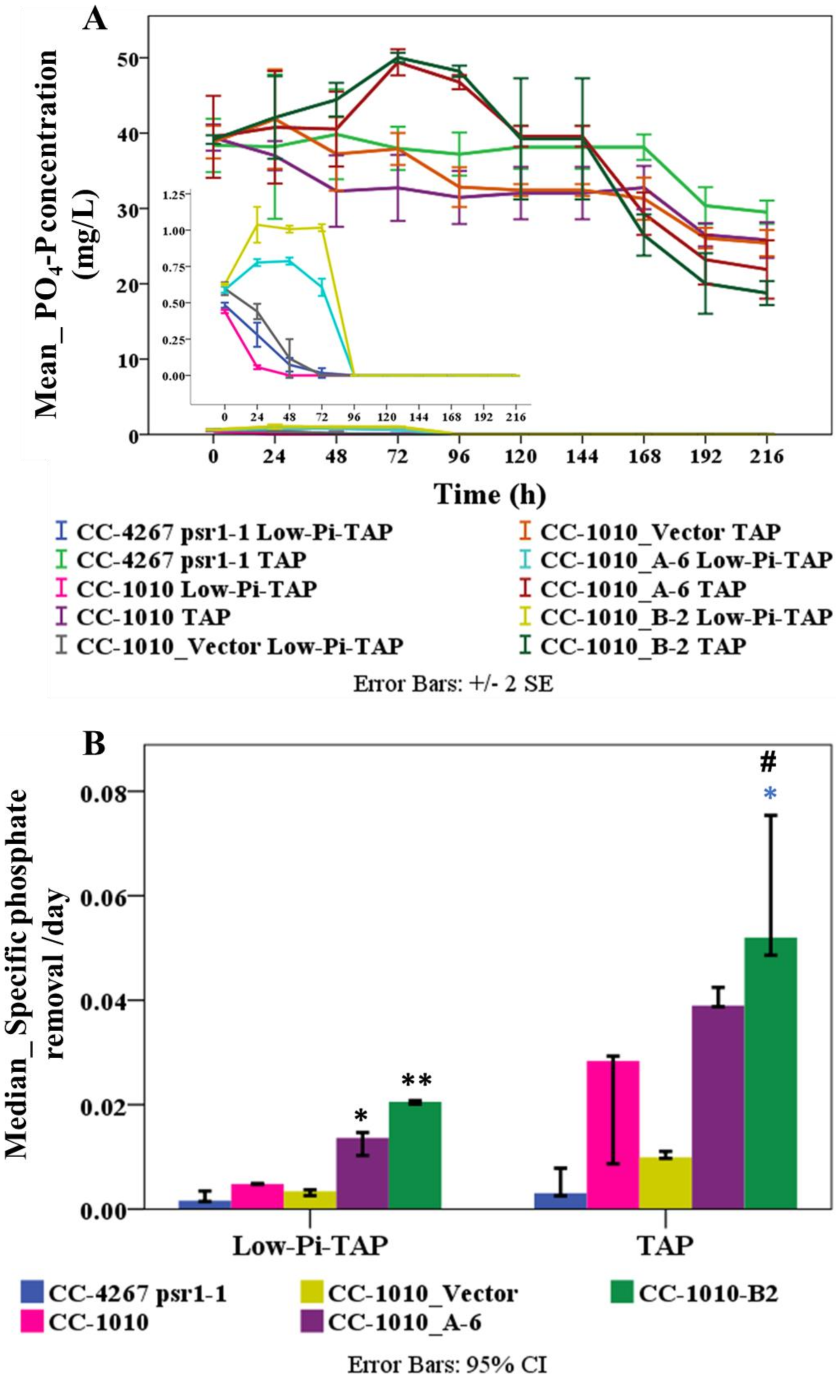

Figure 5.11: The phosphate removal was enhanced in the putative *PSR1* over expression lines. The samples were grown at 70 µmol photon· $m^{-2} \cdot s^{-1}$, L:D 18:6 cycle in hours, 22 °C and mixing at 250 rpm. **(A)** Change in phosphate concentration throughout the experiment: phosphate was removed by the microalgae. (error bars represent standard error; *n* = 3). **(B)** Median of specific phosphate removal per day. The significant difference in the specific phosphate removal rate from the WT in Low-P_i-TAP medium are indicated by black asterisks (*****). Blue asterisks (*) indicate the significant difference in specific phosphate removal rate compared to the WT strain in normal TAP medium. The number sign (#) indicates the significant difference in the specific phosphate removal for the same strain/line in the two types of medium. Samples were statistically analysed using a Kruskal-Wallis test followed by homogenous subsets analysis based on asymptotic significances at the 0.05 significance level (error bars represent 95% confidence intervals; *n* = 3).

Phosphorus content in the investigated microalgae

The total phosphorus (organic and inorganic phosphorus) content in the microalgae strains was monitored during the experiment. To study the effect of *PSR1* overexpression on the phosphate pool in the cells, and to ensure that the decrease in the phosphate level in the in the media is due to phosphate uptake by microalgae not due to precipitation. The accumulation of phosphorus in the microalgae was estimated as a percentage of the biomass (wt/wt:µg of P/µg of algal biomass) and evaluated relative to the cell number (µg of P/10^6 cells). The change in the phosphorus content in the algal biomass and cells during the experiment is presented in Figure 5.12 and shows variation in phosphorus content among the strains. The measurements at 48 and 72 hours have been omitted for the samples because during these time points the putative overexpression lines CC-1010_(A-6 and B-2) were bleached. The differences in the total phosphorus content among the microalgae were statistically evaluated at two time points. The first-time point was when the phosphate was depleted (exponential phase) in the Low-P_i-TAP, and the second-time point was at the end of the experiment (stationary phase). Results are summarised in Figure 5.13 and Table 5.1.

At the phosphate depletion point (cells still in their exponential phase) there was a significant difference between the putative overexpressed lines and the WT strain regarding the phosphorus content per million cells in the Low-P_i-TAP and normal TAP medium (Figure 5.13 A). At the same time point for the percentage of the phosphorus in the algal biomass only CC-1010_B-2 had a significantly higher percentage of the phosphorus in the biomass than the WT at Low-P_i-TAP. In normal TAP the putative overexpression lines acted like the WT (Figure 5.13 C). The highest phosphorus percentage at depletion time was for CC-1010_B-2, 1.93% at the normal and 1.62% low-P_i-TAP (Table 5.1). For all strains/lines there was no significant difference between the phosphorus content at the depletion time in the low-P_i-TAP and normal TAP medium except for CC-1010_A-6 where the percentage of phosphorus in the biomass in the normal TAP medium was significantly higher than the percentage of phosphate at the depletion time.

At the end of the experiment, the amount of phosphorus in a million cells showed no difference between all the studied microalgae including the mt in low-P_i-TAP. However, in the normal TAP, the phosphorous content of CC-1010_B-2 was significantly higher than CC-1010_A-6 and both were significantly higher than the WT (Figure 5.13 B).

Comparison of the same microalgae in Low-P_i-TAP and normal TAP, shows the transformed lines (CC-1010_A-6 and B-2) and the WT strain had significantly different phosphorus content in the normal TAP compared to the low-P_i-TAP (Figure 5.13 B). In the case of the percentage of phosphorus in the algal biomass at the end of the experiment (Figure 5.13 D) in the low-P_i-TAP the WT strain had highest phosphate content (0.22%), significantly different from the transformed lines. However, in normal TAP CC-1010_B-2 had the highest phosphorus percentage (2.37%), which was significantly different to WT and CC-1010_A-6. All the microalgae cultures in the normal TAP medium had a higher phosphorus percentage than when they were grown in low-P_i-TAP medium. (Figure 5.13 D and Table 5.1).

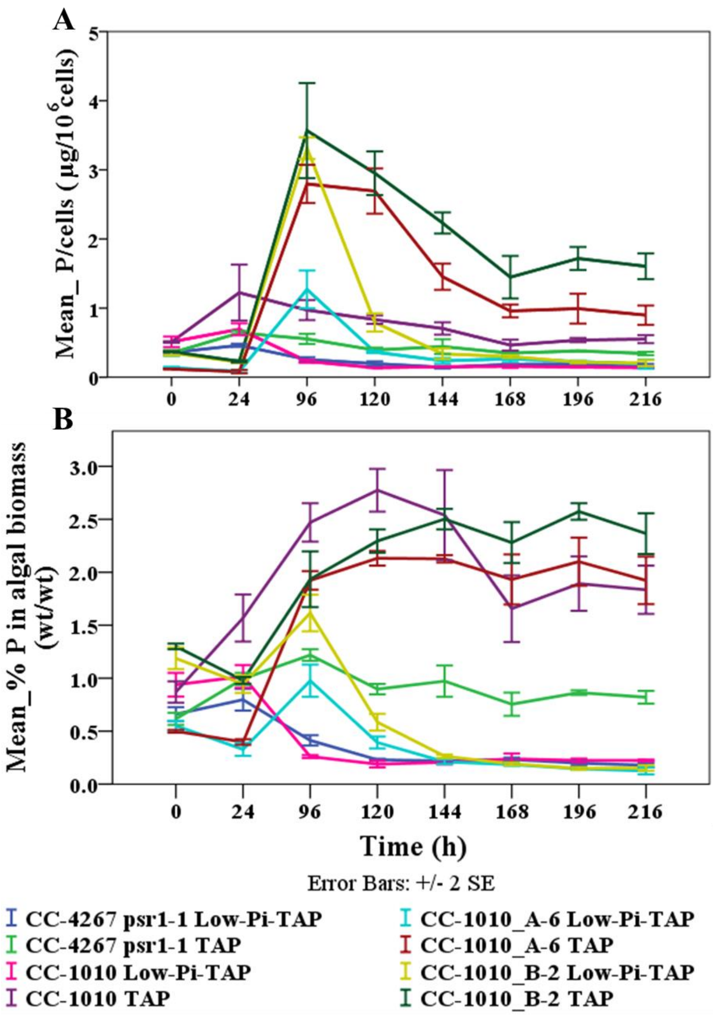

Figure 5.12: Change in total phosphorus content in the algal biomass during the experiment. The samples were grown at 70 µmol photon· m^{-2}·s^{-1}, L:D 18:6 cycle in hours, 22 °C and mixing at 250 rpm **(A)** Change in phosphorus content in the cells during the experiment***.*** ***(*B)** Change in the phosphorus percentage in the algal biomass (Error bars represent standard error; *n* = 3).

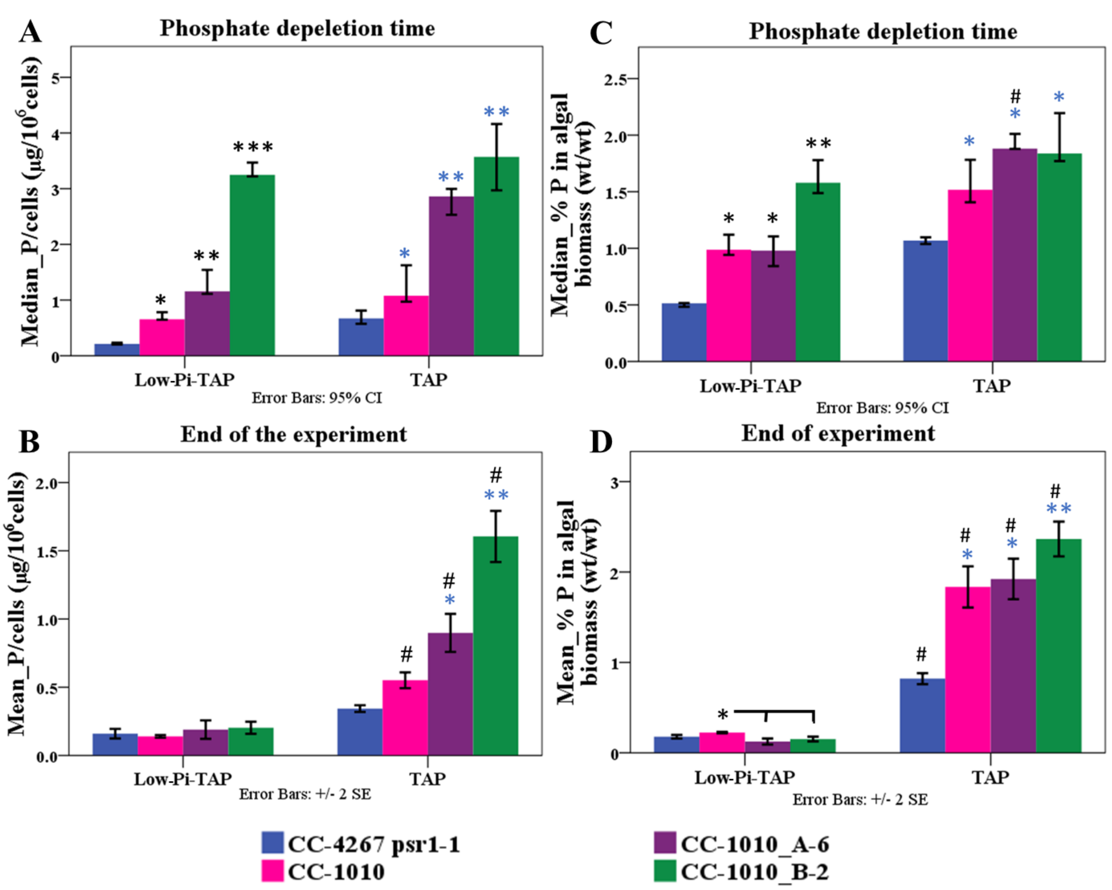

Figure 5.13: Assessment of total phosphorus content in algal biomass at two time points. Microalgae strains/lines were grown in two sets, at Low-P_i-TAP (10 µM PO_4^{3-}) and normal TAP medium with 1mM phosphate, under the following conditions: at 70 µmol photon· m^{-2}·s^{-1}, L:D 18:6 cycle in hours, 22 °C and mixing at 250 rpm. (A) Phosphorus per million cells when the phosphate has been depleted in the low-P_i-TAP medium for both sets of medium (Error bars represents confidence interval at 95%; *n* = 3). **(B)** Phosphorus content per million cells at the end of the experiment (error bars represent standard error; *n* = 3). **(C)** Phosphorus content as a percentage of the biomass at the phosphate depletion time from the low-P_i-TAP (error bars represent 95% confidence intervals; *n* = 3). **(D)** Phosphorus content as a percentage of biomass at the end of the experiment (error bars represent standard error; *n* = 3). Statistical analysis tests were applied: one way ANOVA followed by Tukey's *post-hoc* test (for normally distributed samples) and a Kruskal-Wallis test followed by homogenous subsets analysis based on asymptotic significances (not normally distributed samples). Black asterisks (*) indicate significant differences in low-P_i-TAP strains/lines. Blue asterisks (*) indicate significant differences in strains/lines in normal TAP medium. The number sign (#) indicates a significant difference in the same strain/line at the two TAP media. A significance level of (0.05) was applied.

Table 5.1: Features of the microalgae grown in low phosphate TAP and normal TAP. The average specific growth rate, generation time, phosphate removal rate, percentage of total phosphorus in the biomass and the amount of phosphorus per million cells.

				Depletion	**End**	**Depletion**	**End**
Isolate	*μ*/day	*Tg*	PO_4-P removal rate	%P in algal biomass	%P in algal biomass	μg of P/10^6cell	μg of P/10^6cell
CC-4267 psr1-1 Low-Pi-TAP	0.61	1.14	0.0022	0.50	0.18	0.22	0.16
CC-1010 Low-Pi-TAP	0.91	0.76	0.0048	1.02	0.22	0.69	0.14
CC-1010 vector low-Pi-TAP	0.80	0.87	0.0032				
CC-1010_A-6 Low-Pi-TAP	1.10	0.63	0.0129	0.98	0.12	1.27	0.19
CC-1010_B-2 Low-Pi-TAP	1.12	0.62	0.0205	1.62	0.15	3.31	0.20
CC-4267 psr1-1 TAP	1.11	0.63	0.0045	1.07	0.82	0.68	0.34
CC-1010 TAP	1.02	0.69	0.0221	1.57	1.83	1.22	0.55
CC-1010_Vector TAP	0.98	0.71	0.0102				
CC-1010_A-6 TAP	1.28	0.54	0.0401	1.92	1.92	2.80	0.90
CC-1010_B-2 TAP	1.22	0.57	0.0587	1.93	2.37	3.57	1.60

5.4 Discussion

Large area requirement for HRAPs system is a major challenge, making the WW treatment by microalgae more efficient is an option to reduce the area footprint. Reducing the phosphorus concentration in the WW effluent is a major challenge for WW treatment industry, since the high concentration of phosphorus in the WW is a major reason for eutrophication in lakes and rivers. This chapter studied the possibility of enhancing phosphate removal from the environment by overexpression of PSR1 TF. Two microalgae species where chosen for the improvement *C. reinhardtii* a well-studied microalga, in which the PSR1 was characterised and a *psr1* mt was produced. In addition to Av_12 a promising UK indigenous microalgae isolate for WW treatment.

PSR1 TF was fully amplified and sequenced from *C. reinhardtii* CC-1010 and the sequence was subjected to BLAST to study the variation among the different microalgae species, because the *PSR1* homologous gene is not characterized in Av_12 yet. The MYB DNA binding domain was conserved among the different microalgae species. Which may indicate that the expression of *Chlamydomonas PSR1* may be functional in Av_12. Then *PSR1* sequences for *psr1* mt strain CC-4267 psr1-1 was compared to the WT strain CC-1010 and CC-125 and the expected deletion point mutation in the *PSR1* was confirmed and the growth in TA media was studied too for the confirmation.

Before doing the transformation with pOpt_mVenus_Paro vector which has paromomycin antibiotic as a selection marker. It was essential to determine the appropriate concentration of paromomycin to be used as a selection marker for each microalgae strain. Then the pOpt_mVenus_Paro_PSR1 vector was constructed and linearized to be ready for the transformation by electroporation. The transformation of the *Chlamydomonas strains* with *PSR1* expression vector was successful but not for Av_12.

5.4.1 The cell wall as an obstacle to delivering foreign DNA

DNA delivery by electroporation has been used to transform *C. reinhardtii* and *S. obliquus,* both algal species have a cell wall. Electroporation has been demonstrated to transform cell walled *C. reinhardtii* by Yamano, Iguchi and Fukuzawa (2013) and Ngan et al. (2015) and has been reported for *S. obliquus* (Guo et al. 2013). *Chlorella vulgaris*

(Chow and Tung, 1999) and *Nannochloropsis sp.* (Kilian et al. 2011) were successfully transformed by electroporation, as were the diatoms *Phaeodactylum tricornotum* (Niu et al. 2012; Miyahara et al. 2013) and *Chaetoceros gracilis* (Ifuku et al. 2015) containing silica-based cell walls using a multi-pulse electroporation approach.

The principle of electroporation is to apply a pulse of electric current to cause the formation of transient holes in the cell membrane; the efficiency of this method is affected by temperature, duration of the pulse, field strength, electric conditions, DNA concentration and cell density (Leon and Fernandez, 2007, pp.1-11). Although transforming *Chlamydomonas* by electroporation was successful as shown in Section 5.3.4, the method failed to transform *S. obliquus* Av_12 despite employing a variety of electroporation conditions, buffers and cell densities. Since Av_12 is an environmental isolate collected from a WWTP, selection may have occurred for a modified cell wall that whilst providing greater protection against harsh environmental conditions and toxins, also present an enhanced barrier to DNA uptake via electroporation. In support of this idea, the cell wall of algal cells is known acts as a physiological barrier preventing the transformation cells with foreign DNA (Doron, Segal and Shapira, 2016). The cell wall of *S. obliquus* has a multi-layered structure ultrastructurally similar to that of *C. reinhardtii*. (Voigt et al. 2014). *S. obliquus* strain 633 has inner wall layers containing cellulose, algaenan trilaminar outer layers that contribute to the rigidity of the of these cell walls (Brunner and Honegger, 1985; Burczyk, 1987; Derenne et al. 1992; Blokker el al. 1998; Voigt et al. 2014), and glucosamine-containing biopolymers and glycoproteins (Burczyk et al. 1999; Voigt et al. 2014).

There are a number of other transformation methods that could be applied to circumvent the issues experienced with Av_12. One of the most widely used methods for transformation of microalgae with a cell wall is microprojectile bombardment (biolistic) (Doron, Segal and Shapira, 2016). In this method, tungsten or gold particles (0.5–1.5 μm) are coated with DNA and shot into the cells at high speed (Leon and Fernandez, 2007, pp.1-11). It has been used for the nuclear transformation of *C. reinhardtii* since 1989 (Kindle et al. 1989; Day et al. 1990; Bajhaiya et al. 2016) and for many diatoms including *Phaeodactylum tricornotum* (Apt, Grossman and Kroth-Pancic, 1996; Falciatore et al. 1999), *Cylindrotheca fusiformis* (Fischer et al. 1999; Poulsen and Kröger, 2005), *Navicula saprophila*, and the centric diatoms *Cyclotella cryptica* (Dunahay, Jarvis and Roessler, 1995). Although not attempted in the current work, the microprojectile bombardment may overcome the cell wall obstacle in Av_12 transformation, but will need optimisation to avoid cell damage and achieve a high transformation efficiency.

Agrobacterium tumefaciens mediated transformation has also met with success in transforming cell-walled microalgae, such as *C. reinhardtii* (Kumar et al. 2004). *Agrobacterium* mediated transformation protocols have also published for *Scenedesmus almeriensis* (Dautor et al. 2014) and the green microalgae *Tetraselmis chuii* (Ubeda-Minguez et al. 2015). *A. tumefaciens* is a gram-negative bacteria that usually infects plants causing crown gall disease by transferring the T-DNA from the tumour inducing plasmid (Ti plasmid) to the genome of the plant. This gene transfer is initiated by virulence genes in response to phenolic compounds such as acetosyringone released by plant cells when subject to damage (Gelvin, 2000). In this method pH, temperature and the relation between acetosyringone concentration and co-culturing period (bacteria and algae) need to be optimised (Ubeda-Minguez et al. 2015).

5.4.2 Selection marker for Av_12

An important component of many transformation protocols is a selectable marker. Some of the commonly used selection markers are auxotrophic complementations of the phenotypes such as nitrate reductase or are based on antibiotic resistance genes (Lauersen, Kruse and Mussgnug, 2015). In this study, an antibiotic resistance marker was chosen because we were studying phosphate uptake and therefore avoided interfering with nutrient uptake that would be required for auxotrophic complementation. Various antibiotic selection markers are used for *C. reinhardtii* including aminoglycoside (3′) phosphotransferases *aphVII* from *Streptomyces hygroscopicus* which confers resistance to hygromycin B (Berthold, Schmitt and Mages, 2002), *aphVIII* from *Streptomyces rimosus* conferring resistance to different aminoglycoside antibiotics including paromomycin (Sizova, Fuhrmann and Hegemann, 2001), the phleomycin resistance *ble* gene from *Streptoalloteichus hindustanus* conferring resistance to phleomycin derived antibiotics such as zeocin (Stevens, Purton and Rochaix, 1996). These antibiotic resistance genes have a high GC content and codon bias similar to that of the nuclear genome of *C. reinhardtii,* and were therefore not modified to account for codon bias (Lauersen, Kruse and Mussgnug, 2015). In 2015 the *tetX* gene was synthesised for a NADP-requiring oxidoreductase that hydroxylates tetracycline substrates, conferring resistance to tetracycline for the first time in *C. reinhardtii* (Garcia-Echauri and

Cardineau, 2015). The present study used the *aphVIII* gene in the pOpt_mVenus_Paro vector that confers resistance to paromomycin and was modified from that used by Sizova, Fuhrmann and Hegemann (2001). A small part (ca. 65 nt) was removed without affecting its function (Lauersen, Kruse and Mussgnug, 2015). Paromomycin inhibits mRNA translation in sensitive cells by binding to the tRNA decoding A site of the 16S ribosomal RNA (Vicens and Westhof, 2001).

The sensitivity of *C. reinhardtii* to paromomycin has been studied and the concentration generally used is reportedly 10 mg/L (Sizova, Fuhrmann and Hegemann, 2001; Lauersen, Kruse and Mussgnug, 2015). This concentration is 2 folds greater than the minimum inhibitory concentration (MIC), which is defined as the lowest concentration of an anti-microbial, such as an antibiotic, that inhibits visible growth of a microorganism after an overnight culture (Andrews, 2001). It was reported that *aphVIII*, under the same fused promotor, can confer an increased paromomycin resistance of 2–10 fold in *C. reinhardtii*, making it a suitable selection marker. However, G418 (geneticin), kanamycin and neomycin have a very narrow range of resistance only 1.2–1.3 fold higher than the MIC, and therefore have been avoided as selection markers (Sizova, Fuhrmann and Hegemann, 2001; Garcia-Echauri and Cardineau, 2015).

Díaz-Santos et al. (2013) studied the sensitivity of some marine and freshwater microalgae to paromomycin at concentrations between 0–300 mg/L (*C. reinhardtii*, *Picochlorum sp.*, *Botrycoccus braunii*, *Tetraselmis suecica*, *Chlorella sorokiniana* and *Dunaliella salina*). Paromomycin inhibited the growth of the tested microalgae at a concentration between 30–200 mg/L except for *Dunaliella salina*. This could be due to the interference of saline with paromomycin (Díaz-Santos et al. 2013). For *C. sorokiniana* and *B. brauni*, growth was inhibited by paromomycin at a concentration of 100 mg/L, whilst the growth of *Picochlorum sp*. or *T. suecica* was inhibited at concentrations of 200 mg/L. These results demonstrate that paromomycin resistance varies between different microalgae.

In this study, Av_12 is an environmental isolate from Avonmouth WWTP identified by DNA barcoding as *S. obliquus* (section 3.3.3). The paromomycin sensitivity of this microalgae was compared to three strains of *C. reinhardtii* (CC-1010, CC-125 and CC-4267 psr1-1) using spot tests where approximately 10,000 cells were spotted onto agar plates with different concentrations of paromomycin. The growth of all *C.*

reinhardtii strains was inhibited at 5 mg/L paromomycin, which agrees with the literatures as mentioned earlier (Sizova, Fuhrmann and Hegemann, 2001; Lauersen, Kruse and Mussgnug, 2015). In the case of Av_12, significant growth inhibition was achieved at much the higher concentration of 100 mg/L. In liquid media at concetration of 100 mg/L, 1 % of the population survived after 7 days. During the transformation experiment growth was apparent on the negative control plates at 100 and 200 mg/L paromomycin. When 300 mg/L paromomycin was applied, no growth was observed in the negative control. Following these results, 300 mg/L paromomycin was chosen for selection in the transformation trial. This difference from the spot test could be due to the much higher density of the cells (100–200 times higher) during transformation.

The concentration of paromomycin could be a reason for the failure of transformation trials for Av_12. It could be the case that the *aphVIII* gene can confer a very narrow range of resistance above the MIC, which would make it unsuitable as a marker for Av_12 as is the case for G418, kanamycin and neomycin and *aphVIII* gene in *Chlamydomonas* (Sizova, Fuhrmann and Hegemann, 2001; Garcia-Echauri and Cardineau, 2015).

To check if that the antibiotic caused the failure of the transformation of Av_12, different vectors with different antibiotics could be used. In 2013, Guo and colleagues optimised a method for transforming *S. obliquus* FSP-3 using electroporation. The sensitivity of *S. obliquus* FSP-3 to five different antibiotics was evaluated and showed the strain was resistant to 1000 mg/L ampicillin and streptomycin, whilst kanamycin inhibited its growth only above 300 mg/L. However, gentamicin and chloramphenicol partially inhibited growth at 50 mg/L, which in the case of chloramphenicol inhibition was complete 100 mg/L (Guo et al. 2013)**.** Chloramphenicol could therefore be used as selectable marker for Av_12 in which the case the chloramphenicol acetyltransferase (*CAT*)gene might prove suitable (Geng et al. 2004; Niu et al. 2011; Guo et al. 2013)

5.4.3 Cell bleaching as a response to phosphate starvation stress

Cell bleaching was noticed during these experiments in the WT (section 5.3.1) and overexpression lines (section 5.3.7). The first case occurred when the strain CC-1010 was grown in TA media to determine if it would grow better than the *psr1* mt strain. According to Wykoff et al. (1999) the strain of *C. reinhardtii* (CC-125)—which is different to the strain that we grew (CC-1010)—divided 3 to 4 times whilst the mt divided only once. However, in this experiment we observed bleaching and a reduction in cell number for the WT strain in the first 48 hours, after which it started to recover. The *psr1* mt did not divide at all and the cell number started to decrease when they were grown in phosphate depleted medium. However, these data should be treated with some caution since the experiment was only repeated twice, because the main purpose was to differentiate between the WT and the mt, which was in any case confirmed at the molecular level (Section 5.3.1 Figure 5.1).

The second time bleaching was observed during the phenotyping experiment to characterise the change in the phenotype of the putative *PSR1* overexpression lines, WT and mt. Bleaching and cell death occurred in the overexpression lines and was associated with washing with TA medium, which does not contain phosphate. The process of washing took around three hours between washing and resuspension in TA to distribute the samples in different replicates. It was surprising that only the overexpression lines experienced bleaching and a reduction in cell number due to cell death in the first 48-72 hours before recovering both in terms of becoming green and cell division.

How might nutrient limitation explain these observations? A nutrient limitation response could be specific or general. The specific response to nutrient deprivation is associated exclusively with the limitation of a single nutrient. For example, sulfur starvation is associated with activities that promote sulfur scavenging from the environment (production of SO_4^{2-} transporters or hydrolytic enzymes that free SO_4^{2-} from organic compounds) and recycling of intracellular sulfur (Ferreira and Teixeira, 1992; Grossman et al. 2010, pp.307-348)

A general response to nutrient limitation could occur under a number of different environmental stresses such as: cessation of cell division, accumulation of storage carbohydrate or lipids and decline in photosynthetic activity (Collier and Grossman, 1992; Chang et al. 2005; Grossman et al. 2010, pp.307-348). During nutrient limitation for sulfur, phosphorus or nitrogen the photosynthetic activity in *Chlamydomonas* declines through a variety of mechanisms, and the response to nutrient stress could be bleaching of algal cells. The bleaching response has been reported for nitrogen, sulfur, and phosphorus deprivation, and to heat and light stress (Banaszak et al. 2003). Under nitrogen starvation conditions, *Chlamydomonas* became bleached and altered metabolic activities were noticed such as a reduction in photosynthesis as a result of the loss of electron transport components such as the cytochrome b_6f complex and light-harvesting proteins (Plumley and Schmidt, 1989).

Algal bleaching due to phosphate starvation has been reported by Rosset et al. (2017). They demonstrated that when corals experienced an imbalance in the ratio of nitrogen to phosphorus due to growth in high nitrogen and low phosphate conditions, the phosphate limitation severely disturbed the symbiosis (coral and the dinoflagellate zooxanthellae) and there was a decrease in the biomass of coral and malfunctioning of algal photosynthesis and bleaching of corals. The accumulation of uric acid crystals was an indication of a high nitrogen to phosphate ratio.

In another case of high nitrate and low phosphate conditions, it was reported that phosphate starvation of algal symbiont made corals more vulnerable to heat and light stress-induced bleaching (Wiedenmann et al. 2013). This bleaching effect was linked to the limitation of phosphorus under the high demand of a proliferated algal population. In the symbiont alga Zooxanthellae, phosphate starvation caused a decrease in the efficiency of photosynthesis combined with changes in the ratio of phospho-sulfo-lipids in thylakoid membranes (Wiedenmann et al. 2013). Thylakoid membranes (Figure 5.14) contain two neutral galactolipids as major components: monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), a sulfolipid (sulfoquinovosyldiacylglycerol; SQDG), phospholipids (phosphatidylglycerol; PG), prenylquinones (plastoquinone, tocopherols), and pigments such as chlorophyllsand carotenoids (Douce and Joyard, 1996, pp.69-101; Frentzen, 2004).

The change in the ratio of phospho- to sulfo-lipids in the algal thylakoid membrane could offer a good explanation for the malfunction in photosynthesis under phosphate depletion stress conditions (Wiedenmann et al. 2013). In photosynthetic organisms, the thylakoid membrane needs a sufficient amount of anionic membrane lipids to work properly. Under phosphate depleted conditions the phospholipids (PG) are replaced by sulfolipids (SQDG) to a certain extent to maintain membrane function in photosynthesis (Frentzen, 2004; Wiedenmann et al. 2013). A sufficient level of PG is extremely important for the structure and the function of thylakoid membranes in plants and cyanobacteria (Frentzen, 2004). It has been demonstrated that PG interacts with protein-pigment complexes to achieve their native conformation (Figure 5.14; Frentzen, 2004). In *Chlamydomonas* this strategy of replacing the phosphate with sulfur has been reported during phosphorus deficiency, PG was reduced by roughly 50% in concert with an increase in sulfolipids (Merchant and Helmann, 2012).

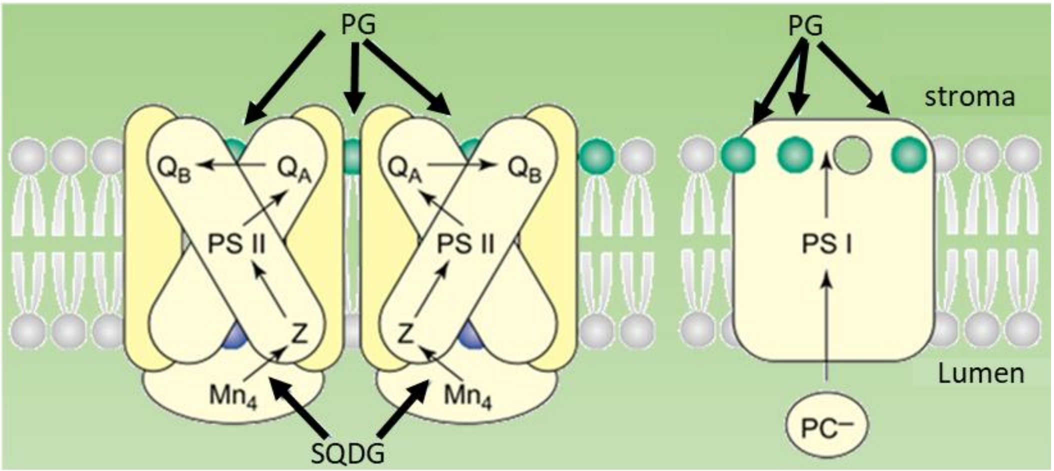

Figure 5.14: Thylakoid membrane components. The arrangement of phosphatidylglycerol, (PG; dark green head group) and in certain organisms, sulfoquinovosyldiacylglycerol (SQDG; blue head group) in a thylakoid membrane, and their interaction with photosystems I (PS I) and II (PS II). Lipid interactions help to preserve the proper dimeric conformation of PS II. Three PG molecules and one molecule of monogalactosyldiacylglycerol (MGDG; light green head group) are integral components of the stromal side of PS I. Grey lipid heads represent neutral galactolipids such as digalactosyldiacylglycerol (DGDG), plastoquinone (QB) and plastoquinone (QA), and plastocyanin (PC; adapted from Frentzen, 2004).

A possible explanation for the bleaching in the *PSR1* overexpression lines is the imbalance in the ratio of sulfo- to phospho-lipids in the thylakoid membranes. The PSR1 TF participates in the synthesis of sulfolipids in the thylakoid membrane during phosphate starvation conditions (Bajhaiya et al. 2016). Bajhaiya et al. (2016) performed RNA sequencing analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the strain CC-125 and the *psr1* mt CC-4267 psr1-1 in low and high phosphate conditions. They reported that phosphate starvation induced the transcripts of the enzymes *SQD1* and *SQD3* —essential for the synthesis of the sulfolipid (SQDG)—in the WT, whereas they were misregulated in the *psr1* mt. This indicates that the PSR1 TF participates in the synthesis of sulfolipids in thylakoid membranes in phosphate starvation conditions. The overexpression of *PSR1* when the cells were washed with phosphate-free TA medium for approximately 3 hours could also have imbalanced the ratio of sulfo- to phospho-lipids, resulting in the bleaching of these strains.

5.4.4 The phosphate removal was enhanced in *PSR1* putative overexpression lines

Our aim was to improve the bioprospected WW indigenous microalgae Av_12 to enable more rapid phosphate removal from WW to reduce the area required for HRAPs and the retention time for WW treatment. It was therefore important to measure the efficiency of phosphate removal in the different tested strains, and establish whether the putative overexpressing *PSR1* enhanced phosphate removal at high and low phosphate concentrations. The results are presented in figure 5.11, which shows that under low phosphate conditions the specific phosphate removal rate from the medium for the putative over expression lines was significantly higher than the WT strain, the mt and the control WT with empty vector. Which could mean that the over expression of *PSR1* enhances the phosphate removal by microalgae from WW at low phosphate conditions, making it more competitive with other microalgae species and bacteria. This is supported by reports that bacteria at low phosphate concentration are more efficient in phosphate uptake (Jansson 1988; Jansson 1993). This would make it easier for our microalgae to compete with other microorganisms on low concentration nutrients in the WW.

In normal TAP, which has a high concentration of phosphate, the CC-1010_B2 overexpression strain had a significantly higher specific phosphate removal rate than the WT. The putative overexpression of *PSR1* was seen to enhance phosphate uptake in *C. reinhardtii* CC-1010_B2 at high and low phosphate conditions (Figure 5.11). It worth to noting again that the phosphate concentration in low-P_i-TAP, normal TAP, Somerton WW, Jordan WW in this study were around (0.4mg/L, 40 mg/L, 2 mg/L, 5 mg/L) respectively. To confirm that the removed phosphate was taken up by the microalgae, the total phosphorus in the algal cells was also measured. Figure 5.15 shows the performance of phosphate removal, phosphorus content in the biomass and growth.

5.4.5 The phosphate concentration in the media was increased at the beginning of the experiment

To explore the difference in behaviour of microalgal strains, the growth, phosphate removal and phosphorus content curves for each alga during the experiment were plotted together in Figure 5.15. For the overexpression lines during bleaching, the phosphate concentration in the media increased. This increase could be explained by the first supposition is the release of phosphate from the dead algal cell (Uehlinger, 1986). Figure

5.15 shows that there was a decline in growth, indicated by the ln of the DW content in the first 48 hours for the putative overexpression lines. This was confirmed by the numbers of the DW content, which show a decrease in the biomass in these lines ranging from 19%–28% (Table 5.2). Cell death could release some phosphorus to the media but what was noticed was that the decrease in biomass did not correlate with the increase in phosphate in the medium. For example, the decrease in biomass in the low phosphate TAP and the normal TAP is very close (20.4% and 19%), but the increase in phosphate was much greater in the TAP 10 mg/L than the low-P_i-TAP 0.39 mg/L. This observation indicates that there may be other factors at play.

The second supposition is that the microalgae release some phosphates to the medium. Phosphorus excretion from microalgae cells as inorganic phosphate and organic phosphorus has been reported by (Lean, 1973; Lean and Nalewajko, 1976; Jansson, 1993). Organic phosphorus has been reported to be excreted in phosphate limited conditions (Lean, 1973; Lean and Nalewajko, 1976) and orthophosphate excretion in high phosphate conditions has been reported by (Jansson, 1993). The reason behind phosphorus excretion is not quite clear. It has been proposed that this occurs as a means of regulating phosphorus concentrations in cells (Lean, 1973; Lean and Nalewajko, 1976; Jansson, 1993). In this experiment the phosphate secretion hypothesis could be supported by the decrease in the total phosphorus content of the cells (Figure 5.15). There was a decrease in the total phosphorus content in the algal biomass (20%–40%) after 24 hours table 5.2. Again, the degree of decrease in phosphorus content in the cells did not correlate with the high increase in phosphate content in the TAP medium and the small increase in the Low-P_i-TAP medium. It is very clear in the overexpression line B-2 that the initial biomass and the percentage of phosphorus decreasing in the biomass are very close to each other but the phosphate increase is very different Table 5.2.

Table 5.2: Changes in putative overexpression lines DW and phosphorus

Microalgae	DW (mg/L) t = 0	Decrease_DW t = 48	Decrease_%P t = 24	Increase_PO_4^{3-} medium
CC-1010_A-6 Low-P_i-TAP	25	28%	40%	0.2 mg/L
CC-1010_A-6 TAP	24	20%	20%	10.4 mg/L
CC-1010_B-2 Low-P_i-TAP	35.7	20.4%	23%	0.39 mg/L
CC-1010_B-2 TAP	34.8	19%	25%	10.9 mg/L

The third supposition is that under high phosphate concentration the bleached algae cells excreted a compound the reacted with the high phosphorus concentration in the medium and this compound interferes with phosphate reaction test such as (P_2O_4). However, there is no evidence to support this hypothesis.

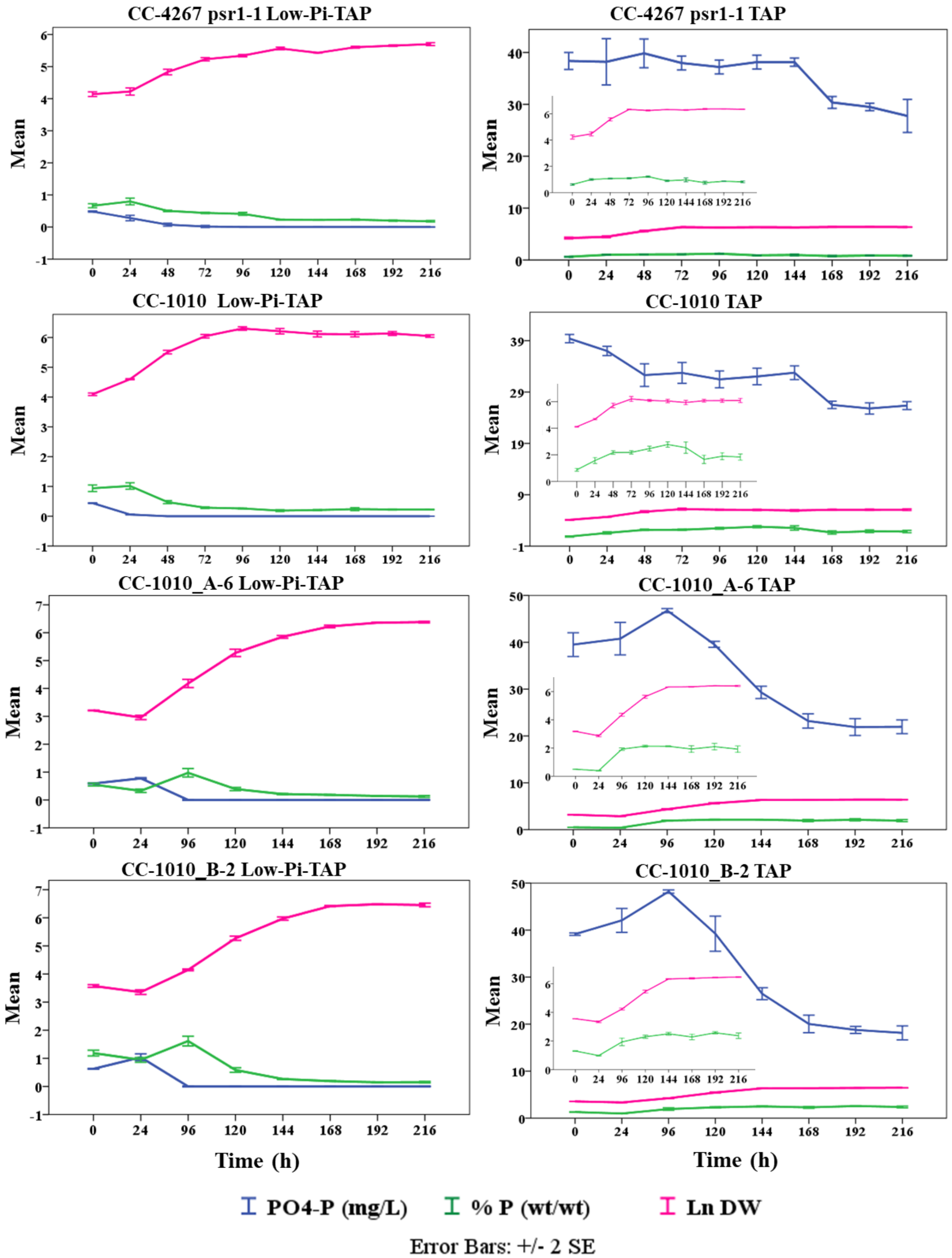

Figure 5.15: Assemble of phosphate in media, total phosphorus in the microalgae and the growth rate for each microalga line during the experiment. Each microalgae line was grown in Low-P_i-TAP and in normal TAP, each graph summarizes the behaviour of the phosphate removal from the media in the meantime the total phosphorus percentage in the algal biomass (P µg / DW µg) and the growth of the microalgae. Error bars represent standard error (n = 3), in some cases the error bars are too small to see but they are present. In the putative overexpression lines CC-1010_(A-6 and B-2) the time 48 and 72 hours were skipped because of the bleaching problem.

5.6 Conclusions and future work

One way to reduce the large area requirement of the HRAPs, for WW treatment is by enhancing the efficiency of the microalgae in nutrient removal. Since the phosphorus a problematic element in WW treatment. This chapter aimed to establish a protocol for enhancing phosphate removal by *PSR1* overexpression in *C. reinhardtii* as well studied microalgae in parallel with same attempts to be applied for Av_12, a promising indigenous strain for WW treatment was isolated from Avonmouth WWTP and applied in HRAPs system in Beckington WWTP.

The PSR1 TF was amplified and sequenced from the *Chlamydomonas* WT strain CC-1010 and mt CC-4267 psr1-1 to confirm the difference at the molecular level for these studied strains. A construct containing the *PSR1* gene amplified from the CC-1010 strain was then built. *Chlamydomonas* transformation by electroporation was optimised successfully, but unfortunately, the transformation of Av_12 was not successful by electroporation. This failure could have occurred because the cell wall acts as a barrier. If this is the case it would be useful to try another method for transformation like biolistic bombardment. Another possible reason is that Av_12 has a very narrow range of paromomycin resistance higher the MIC. Since Av_12 is more resistant to paromomycin than *Chlamydomonas*, paromomycin may be not useful as a selection marker for Av_12. In the future, it could be useful to try another selection marker.

To determine whether overexpression of *PSR1* enhanced phosphate removal, the differences in growth, phosphate removal and phosphorus content for the two overexpression lines, WT and the mt, were studied in Low-P_i-TAP and normal TAP. The growth in the low-Pi-TAP for the putative overexpression lines and the WT strain was the same, but in normal TAP CC-1010_A-6 was the fastest growing with a significant difference from the WT and the rest of the strains. For the phosphate removal CC-1010_B-2 performed the best with a significant difference from the WT and other strains in both low-P_i-TAP and the normal TAP and this may be proof that manipulation of *PSR1* could enhance phosphate removal for WW treatment. At the end of the experiment the CC-1010_B2 in normal TAP had a higher phosphate content than the WT but not at low-P_i-TAP.

In the future, performing qRT-PCR to confirm that *PSR1* is actually overexpressed in comparison with the WT would support its role in the change in the phenotypes. I would recommend repeating the phenotyping experiment with a washing step with TA and without a washing step to study of the bleaching of the overexpression lines. It would also be interesting to determine the effect of the overexpression of *PSR1* on the lipid and carbohydrate accumulation in the microalgae. It has been reported previously to increase the lipid content in *C. reinhardtii* WT strain 4a+ (Ngan et al. 2015), and to increase the carbohydrate content in *C. reinhardtii* strains cw15 arg7-8, and *psr1* mt CC-4267 psr1-1 (Bajhaiya et al. 2016).

Chapter 6
Final Conclusions

Final conclusions

In this study, parallel work was performed with samples from the UK and Jordan, thereby offering two very different climates, and water and energy resource situations. Although the UK is a developed country while Jordan is a developing county, both require sustainable WW treatment methods. In the case of the UK, improvements in WW treatment are essential in order to avoid eutrophication, which causes environmental and economic problems. In contrast, in Jordan, as a water scarce country, the WW effluent is an important source of recycled water to reduce the pressure on fresh water resources. This is mainly achieved by using the WW effluent in agriculture, which consumes approximately 60% of Jordan's fresh water resources.

Microalgae offer a promising environmentally friendly method for WW treatment. This method, however, still needs to be optimized if it is to be applied successfully in large scale open ponds at low cost and with a small area footprint for WW treatment in different seasons and different parts of the world. This research is a part of a project that aims to apply microalgae for efficient WW treatment in large scale open ponds (HRAPs). The research aimed to tackle several problems regarding the large scale use of microalgae for WW treatment, such as adaptation to different local environments taking into account factors such as the WW composition, light intensity and duration, and temperature. In this context, a bioprospecting process was carried out in WWTPs in the UK and Jordan for indigenous microalgae, which were then screened in order to evaluate their efficiency for use in WW treatment, specifically in terms of efficient nitrogen removal, phosphate removal and biomass production. Another issue that was considered was how to reduce the costs involved in the harvesting the microalgae. To this end, the microalgae isolates where evaluated for harvesting by gravity. Furthermore, the study sought to improve the efficiency with which the microalgae were able to remove phosphate so as to reduce the area foot print of the WW system.

Bioprospecting for indigenous microalgal species.

The idea of using microalgae in open ponds for WW treatment started around 60 years ago with the work of Oswald and Gotaas (1957). For a successful algal-based WW treatment system and biomass production, it is important to choose the strain(s) that are to be used carefully, since they will be applied in open ponds where it is impossible to control the environmental conditions and thus where there is a high possibility of invasion by predators and pathogens. It is therefore essential to bioprospect for strains that are already adapted to their target environments so that can tolerate changes in the conditions, predation and pathogens. Thus, samples were collected from WWTPs in the UK and in parallel in Jordan

After molecular characterization using 18S rDNA and ITS1-5.8S-ITS2 markers, eight different isolates were obtained from the UK and another eight from Jordan. The microalgae isolates from the UK and Jordan belonged to at least six genera: *Chlorella* (UK; Av_2 and So_3, Jordan; Jo_40 Jordan), *Desmodesmus* (UK; Av_3 and 10, Jordan; Jo_2, 4, 18, 23 and 29), *Scenedesmus* (UK; Av_12, Jordan; Jo_34), *Scotiellopsis* (UK; So_32), *Coelastrella* (Jo_12), and *Monoraphidium* or *Ankistrodesmus* (UK; Av_7 and So_15). The first three genera were common between the two countries, with two confirmed species in common (*S. obliquus* and *C. sorokiniana*). Six of the 16 isolates were identified at the species level and, apart from two from the UK (Av_7 and So_15), the remaining isolates were confirmed at the genus level. This difficulty in microalgae identification occurred because the 18S rDNA is too conserved to differentiate between closely related species (Krienitz and Bock, 2012; Leliaert et al. 2014). Although the ITS1-5.8S-ITS2 sequence is more variable (Leliaert et al. 2014) the poor database of algal DNA in GenBank limited the identification of algae; that is many algal isolates in GenBank are only at the genus or family level, or even only as context (e.g. environmental) samples (Darienko et al. 2015). Furthermore, the entries that are present in the database are not fully representative of all the actual species. Furthermore, changes in algal classification make the molecular identification of the algal isolates hard. For these reasons, a polyphasic approach has been suggested to identify microalgae (Pröschold and Leliaert, 2007, pp. 123-153; Krienitz and Bock, 2012) that combines morphology, ecophysiology and genetic methods. Identifying the microalgae and differentiating between the obtained microalgae isolates is an essential step, but this needs to be followed by a screening process to evaluate the performance of these microalgae isolates for WW treatment.

Assessment of the microalgae isolates performance in treating the WW

The next step in planning to apply the indigenous microalgae isolates for WW treatment is to assess their performance in WW treatment with respect to their growth, nutrient removal abilities and their settleability in real WW. The microalgae from the UK were therefore grown in the secondary effluent of Somerton WW, while the microalgae obtained from Jordan were grown in a WW similar to the average of the annual final effluent from the WWTPs in Jordan. Both, the UK isolates and Jordan isolates were assessed alongside a reference strain *S. obliquus* CCAP 267/7, with CO_2 supplementation in optimum growth conditions. Then the microalgae isolates were given a score depending on their performance and ranked. Some isolates performed better than others under the experimental conditions (i.e. Av_12 and Av_10 from the UK isolates, and Jo_18 and Jo_29 from the Jordan isolates), and these were considered to be good candidates for application for WW treatments. The promising microalgae from the UK (Av_12) was grown on a large scale in order to assess its performance in treating WW. Specifically, Av_12 was up-scaled into 0.5 m^3 raceway ponds in a glass house at the University of Bath and then grown in pilot-scale HRAPs in Beckington, (i.e. in ponds with a capacity of 18 m^3). In future, it is intended to follow the same protocol in Jordan using the Jordan isolates. Work is ongoing using (FTIR) and (NIR) spectroscopy at the Department of Archaeology, Anthropology and Forensic Science at Bournemouth University, to assess the biomass composition of the microalgae isolates obtained from the UK and Jordan and grown in WW. The results of this biomass assessment for the protein, polysaccharides and lipids will help in determining the fate of the algal biomass that is produced from the WW treatment. If it is rich in lipid or polysaccharides it can be used for biofuel production. If it is rich in protein it can be used for animal feed or it can be used as a fertilizer

Figure 6.1: Pilot-scale HRAPs in Beckington WWTPs managed by the University of Bath, where Av_12 was applied for secondary effluent WW treatment.

Improvement of phosphate removal

A major disadvantage of applying microalgae for WW treatment process, in open ponds in large scale is the need for relatively large area for constructing the ponds. This will increase the cost of construction, and it will be hard to apply in urban areas where the land is limited and expensive. Part of this research aimed to reduce the area and time for WW treatment by improving the microalgae ability to remove phosphorus from the WW. The phosphorus was chosen because it is the main cause for eutrophication in the fresh water resources (DEFRA, 2012) and the legal limits for the phosphorus in the final effluent of WWTPs are expected to become more strict.

The PSR1 TF help in scavenging the phosphorus from the environment by inducing the expression of, some of the high affinity phosphorus transporters and the calcium dependent alkaline phosphatase (Moseley, Chang and Grossman, 2006). The hypothesis of this part of the work is the overexpression of the *PSR1* in the microalgae can improve their efficiency to remove the phosphate from the WW. Consequently, over expressing *PSR1* in Av_12 will increase the efficiency of this isolate in removing the phosphate from the WW and reduce the area foot print. To achieve this transformation protocols were carried on for Av_12 and *C. reinhardtii* as a control to proof the effect of the overexpression first on it because the *PSR1* TF that was used for transformation was isolated from *C. reinhardtii* and it was not characterized in Av_12 (*S. obliquus*). Transformation protocols using electroporation were applied on both Av_12 and *C. reinhardtii* to deliver the constitutive expression vector that harbour the *PSR1* TF whole gene.

The transformation trials for *C. reinhardtii* was successful but not for Av_12. due to the limited time, the experiment progressed to phenotype characterization for *C. reinhardtii* putative over expression lines, leaving transforming Av_12 for future work.

The transformation of *C. reinhardtii* was successful for the *Chlamydomonas* strains C-1010_WT. The effect of *PSR1* overexpression on the efficiency of phosphate removal and the specific growth rate was evaluated for the two overexpression lines (CC-1010_A6 and CC-1010_B2) in comparison with C-1010 _WT, CC-1010_WT_vector and CC-4267_mt. The growth of the putative overexpression lines was the same as the growth rate of the wild type in the low P_i-TAP, Whilst, CC-1010_A-6 was the fastest growing with a significant difference among all the strains in normal TAP medium. CC-1010_B-

2 performed the best with a significant difference from the all the strains for the phosphate removal in both low P_i-TAP and the normal TAP. This may be a proof that manipulation of *PSR1* could enhance phosphate removal for WW treatment. But to confirm this at the molecular level. qRT-PCR need to be performed in the future to quantify the expression of *PSR1* in the putative overexpression lines and the wild type *Chlamydomonas* strain.

Concluding remarks

Obtaining a monoculture of different microalgae isolates and differentiate between them and identify the at least at the genus level is an important first step for building data base for microalgae that inhibits WW and can be applied for WW treatment and biomass production. These data will help in understanding the nature and the variation of microalgae that are presented in the WW in the UK and in Jordan. In addition, identifying the microalgae is important to help in understanding the physiology, behaviour and predict its response to different environmental conditions. Supporting these data with information about the specific growth rate, nitrogen removal, phosphorus removal, settleability will provide good base for designing a biological system for WW treatment using microalgae in open pond by applying a single species or even designing algal consortia that will support WW treatment and biomass production. Reducing the harvesting cost and area foot print is a major consideration to reduce the cost of WW treatment. Improving the efficiency of phosphorus removal by microalgae can reduce the time and the area needed for treatment making the process more efficient.

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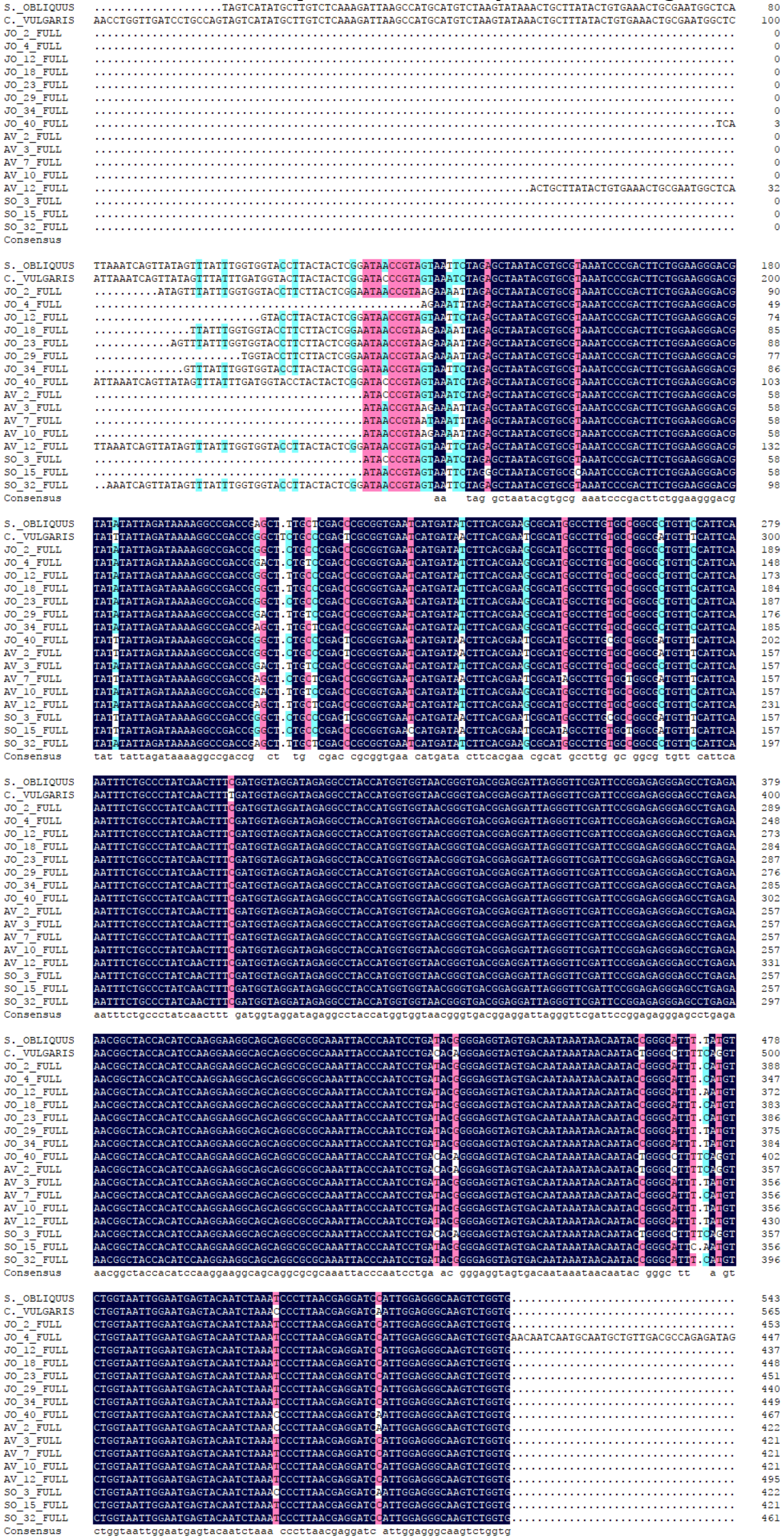
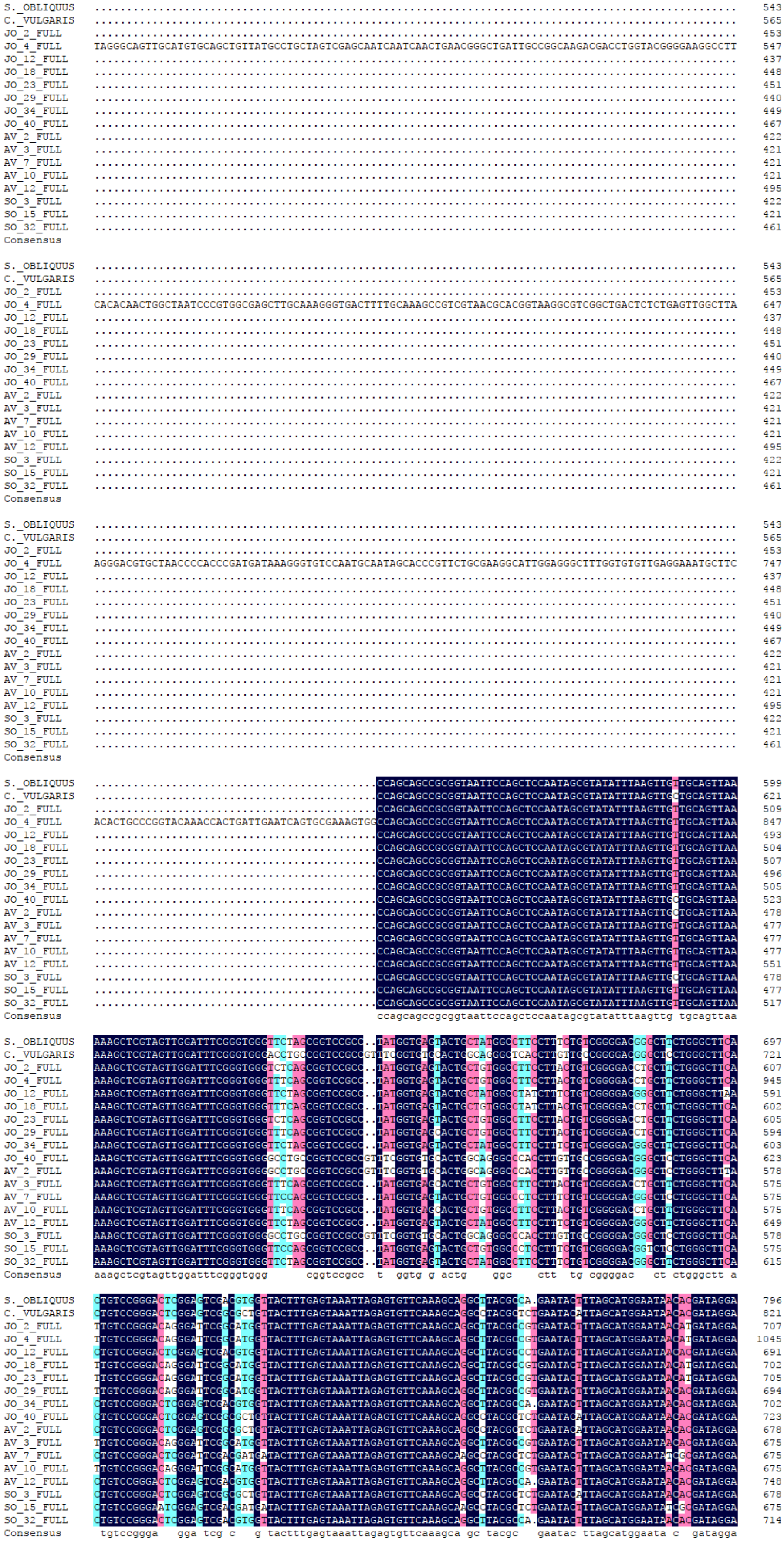
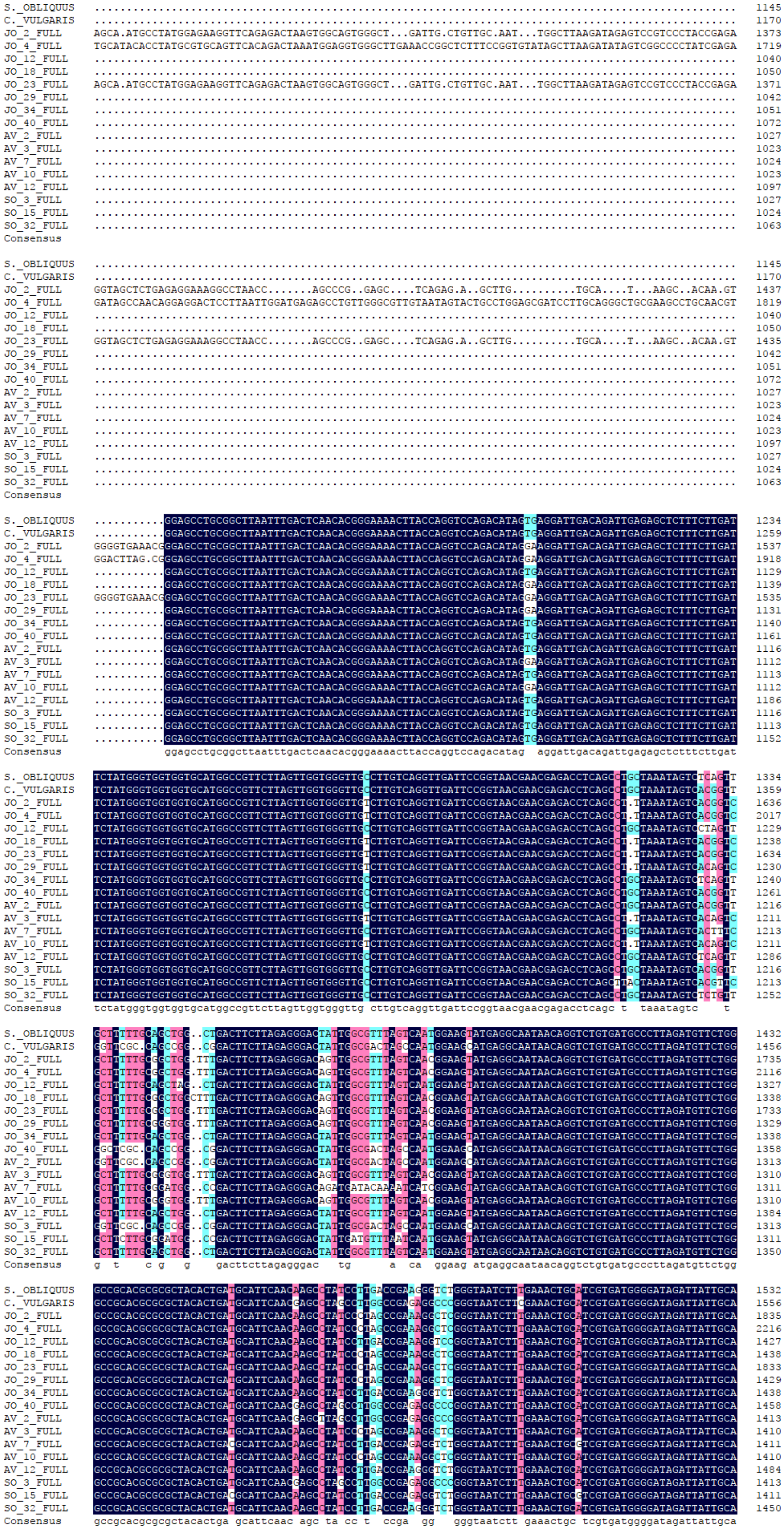
Appendices

Appendix 1

Appendix 1. A: Multiple alignments for the row 18SrRNA sequences of the UK and Jordan isolates and the sequences of *S. obliquus and C. vulgaris* from the GenBank**.**






S._OBLIQUUS
C._VULGARIS
JO_2_FULL
JO_4_FULL
JO_12_FULL
JO_18_FULL
JO_23_FULL
JO_29_FULL
JO_34_FULL
JO_40_FULL
AV_2_FULL
AV_3_FULL
AV_7_FULL
AV_10_FULL
AV_12_FULL
SO_3_FULL
SO_15_FULL
SO_32_FULL
Consensus

```
S._OBLIQUUS   ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1632
C._VULGARIS   ATTATTAATCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1656
JO_2_FULL     ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1935
JO_4_FULL     ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   2316
JO_12_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1527
JO_18_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1538
JO_23_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1933
JO_29_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1529
JO_34_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1538
JO_40_FULL    ATTATTAATCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1558
AV_2_FULL     ATTATTAATCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1513
AV_3_FULL     ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1510
AV_7_FULL     ATTATTAGTCTTCAACGAGGAATGCCTAGTAGGCGCGATTCATCAGATCGCGCCGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1511
AV_10_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1510
AV_12_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1584
SO_3_FULL     ATTATTAATCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1513
SO_15_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAGGCGCCAGTCATCAGCTGGCGCCGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1511
SO_32_FULL    ATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA   1550
Consensus     attatta tcttcaacgaggaatgcctagta gcgc a tcatcag t gcg  gattacgtccctgccctttgtacacaccgcccgtcgctcctaccga

S._OBLIQUUS   TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCAGCTTAGGGTGGCAACACCTCAGGTCTGCCGAGAAGTTCATTAAACCCTCCCACCTAGAGGAAGGAGAA   1732
C._VULGARIS   TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCGACCTGGGGCGGTCTCCGCTCTCGGCCGCCGAGAAGTTCATTAAACCCTCCCACCTAGAGGAAGGAGAA   1756
JO_2_FULL     TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCAA.TTGAAGGTGGCAACACCGTCGATTGCCGAGAAGTTCATTAAACCCTCCCACCTAGAGGAAGGAGAA   2034
JO_4_FULL     TTGGGTGTGCTGGTGAAGTGTTCGGATTGG......................................................................   2346
JO_12_FULL    TTGGGTGTGCTGGTGAAGTGTTC.............................................................................   1550
JO_18_FULL    TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCA....................................................................   1570
JO_23_FULL    TTGGGTGTGCTGGTGAAGTGTTCGGA..........................................................................   1959
JO_29_FULL    TTGGGTGTGCTGGTGAAGTGT...............................................................................   1550
JO_34_FULL    TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCAG...................................................................   1571
JO_40_FULL    TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCGACCGGGGGCG..........................................................   1600
AV_2_FULL     TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCGACCG................................................................   1549
AV_3_FULL     TTGGGTGTGCTGGTGAAGTGTTCGGATTGGTGA.TTG...............................................................   1546
AV_7_FULL     TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCGAAGC................................................................   1547
AV_10_FULL    TTGGGTGTGCTGGTGAAGTGTTCGGATTGGTGA.TTG...............................................................   1546
AV_12_FULL    TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCAGCTTAGGGTGGCAACACCTCA...............................................   1637
SO_3_FULL     TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCGACCG................................................................   1549
SO_15_FULL    TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCAGAGC................................................................   1547
SO_32_FULL    TTGGGTGTGCTGGTGAAGTGTTCGGATTGGCAGCTTAGGGTGG.........................................................   1593
Consensus     ttgggtgtgctggtgaagtgt

S._OBLIQUUS   GTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGAATTATTAAAACCACAATGCGAACCTATCTGTTCCGTGCCTTAGCTGCCAGCAAGG   1832
C._VULGARIS   GTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCAA..........................................................   1798
JO_2_FULL     GTCGTAACAAGGTTTCCGTTGAATCACTAGCGGATAAAAGTCAGCCTGAAAGGGGTGGCTAGTCTTTGTCGGCTCGCTATTGGTGAGAGCCTTCAAAGGC   2134
JO_4_FULL     ....................................................................................................   2346
JO_12_FULL    ....................................................................................................   1550
JO_18_FULL    ....................................................................................................   1570
JO_23_FULL    ....................................................................................................   1959
JO_29_FULL    ....................................................................................................   1550
JO_34_FULL    ....................................................................................................   1571
JO_40_FULL    ....................................................................................................   1600
AV_2_FULL     ....................................................................................................   1549
AV_3_FULL     ....................................................................................................   1546
AV_7_FULL     ....................................................................................................   1547
AV_10_FULL    ....................................................................................................   1546
AV_12_FULL    ....................................................................................................   1637
SO_3_FULL     ....................................................................................................   1549
SO_15_FULL    ....................................................................................................   1547
SO_32_FULL    ....................................................................................................   1593
Consensus

S._OBLIQUUS   CAATTGGCTTGCCCAATTGTACTTGCAAGCTGGTGCGAGTAATTTGATTACTTGCATCAGTGGCGCTTTGGCATGCTTATACACCAGTGCTAACCACTGT   1932
C._VULGARIS   ....................................................................................................   1798
JO_2_FULL     AACACTGTCGAATTGCGGGAAGTTCCTTAGAGCCCTCACTACCAAGCTAGGGTGGAAACACACCTAGTGGCCAGGTTAATTGCCTCGGGTATGGTAAAAA   2234
JO_4_FULL     ....................................................................................................   2346
JO_12_FULL    ....................................................................................................   1550
JO_18_FULL    ....................................................................................................   1570
JO_23_FULL    ....................................................................................................   1959
JO_29_FULL    ....................................................................................................   1550
JO_34_FULL    ....................................................................................................   1571
JO_40_FULL    ....................................................................................................   1600
AV_2_FULL     ....................................................................................................   1549
AV_3_FULL     ....................................................................................................   1546
AV_7_FULL     ....................................................................................................   1547
AV_10_FULL    ....................................................................................................   1546
AV_12_FULL    ....................................................................................................   1637
SO_3_FULL     ....................................................................................................   1549
SO_15_FULL    ....................................................................................................   1547
SO_32_FULL    ....................................................................................................   1593
Consensus

S._OBLIQUUS   CAAAACCAAACTCTGAAGCTTTGATTGCTATTAACTGGCAATCTTAACCAAAGACAACTCTCAACAACGGATATCTTGGCTCTCGCAACGATGAAGAACG   2032
C._VULGARIS   ....................................................................................................   1798
JO_2_FULL     TGTGAGGGATTGGATAATCCGCAGCCAAGCTCCTAAGGGTGGCAACACCTATGGAGAAGGTTCAGAGACTAAGTGGCAGTGGGCTGATTGCCTTGGCAAT   2334
JO_4_FULL     ....................................................................................................   2346
JO_12_FULL    ....................................................................................................   1550
JO_18_FULL    ....................................................................................................   1570
JO_23_FULL    ....................................................................................................   1959
JO_29_FULL    ....................................................................................................   1550
JO_34_FULL    ....................................................................................................   1571
JO_40_FULL    ....................................................................................................   1600
AV_2_FULL     ....................................................................................................   1549
AV_3_FULL     ....................................................................................................   1546
AV_7_FULL     ....................................................................................................   1547
AV_10_FULL    ....................................................................................................   1546
AV_12_FULL    ....................................................................................................   1637
SO_3_FULL     ....................................................................................................   1549
SO_15_FULL    ....................................................................................................   1547
SO_32_FULL    ....................................................................................................   1593
Consensus

S._OBLIQUUS   CAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATTGCGCTCGAGCCCTCGGGCAAGAGCATGTCTGCC   2132
C._VULGARIS   ....................................................................................................   1798
JO_2_FULL     CGGCTTAAGATAGAGTCCGGCCCACCCGAGAG....................................................................   2366
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JO_12_FULL    ....................................................................................................   1550
JO_18_FULL    ....................................................................................................   1570
JO_23_FULL    ....................................................................................................   1959
JO_29_FULL    ....................................................................................................   1550
JO_34_FULL    ....................................................................................................   1571
JO_40_FULL    ....................................................................................................   1600
AV_2_FULL     ....................................................................................................   1549
AV_3_FULL     ....................................................................................................   1546
AV_7_FULL     ....................................................................................................   1547
AV_10_FULL    ....................................................................................................   1546
AV_12_FULL    ....................................................................................................   1637
SO_3_FULL     ....................................................................................................   1549
SO_15_FULL    ....................................................................................................   1547
SO_32_FULL    ....................................................................................................   1593
Consensus
```

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S._OBLIQUUS   TCAGCGTCGGTTTATACCCTCACCCCTCTCTCCTTTTGGAGGGCTGGTCAGCTTCTAGTTGGCCTCAGGGATGGATCTGGCTTTCCCAATTGGTTCACTC   2232
C._VULGARIS   ....................................................................................................   1798
JO_2_FULL     ....................................................................................................   2366
JO_4_FULL     ....................................................................................................   2346
JO_12_FULL    ....................................................................................................   1550
JO_18_FULL    ....................................................................................................   1570
JO_23_FULL    ....................................................................................................   1959
JO_29_FULL    ....................................................................................................   1550
JO_34_FULL    ....................................................................................................   1571
JO_40_FULL    ....................................................................................................   1600
AV_2_FULL     ....................................................................................................   1549
AV_3_FULL     ....................................................................................................   1546
AV_7_FULL     ....................................................................................................   1547
AV_10_FULL    ....................................................................................................   1546
AV_12_FULL    ....................................................................................................   1637
SO_3_FULL     ....................................................................................................   1549
SO_15_FULL    ....................................................................................................   1547
SO_32_FULL    ....................................................................................................   1593
Consensus

S._OBLIQUUS   CGATTGGGTTGGCTGAAGCTTAGAGGCTTAAGCAAGGACCCGATATGGGCTTCAACTGGATAGGTAGCACCGGCTTCTGCCGACTACACGAAGTTGTGGC   2332
C._VULGARIS   ....................................................................................................   1798
JO_2_FULL     ....................................................................................................   2366
JO_4_FULL     ....................................................................................................   2346
JO_12_FULL    ....................................................................................................   1550
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JO_40_FULL    ....................................................................................................   1600
AV_2_FULL     ....................................................................................................   1549
AV_3_FULL     ....................................................................................................   1546
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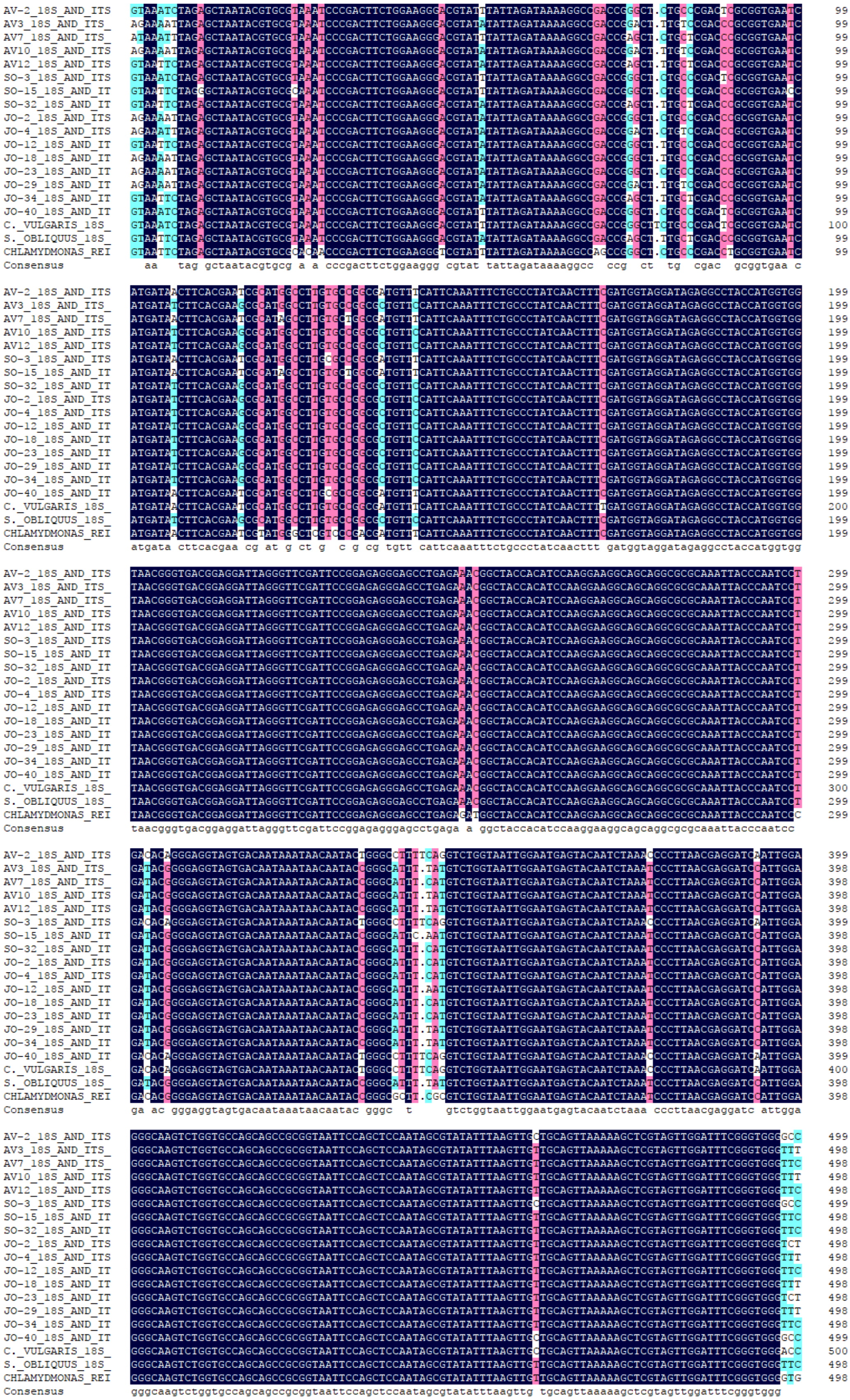
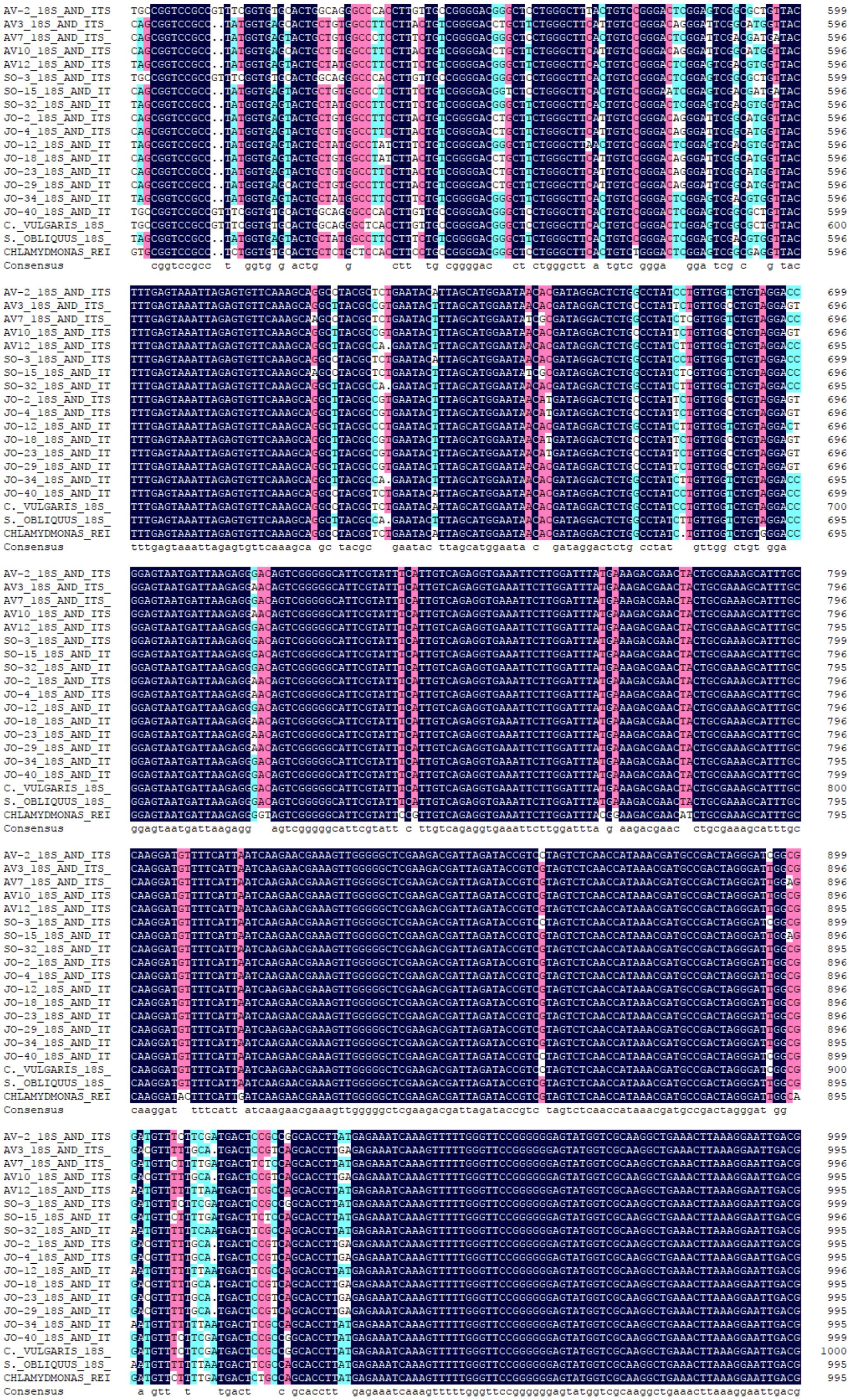
AV_10_FULL    ....................................................................................................   1546
AV_12_FULL    ....................................................................................................   1637
SO_3_FULL     ....................................................................................................   1549
SO_15_FULL    ....................................................................................................   1547
SO_32_FULL    ....................................................................................................   1593
Consensus

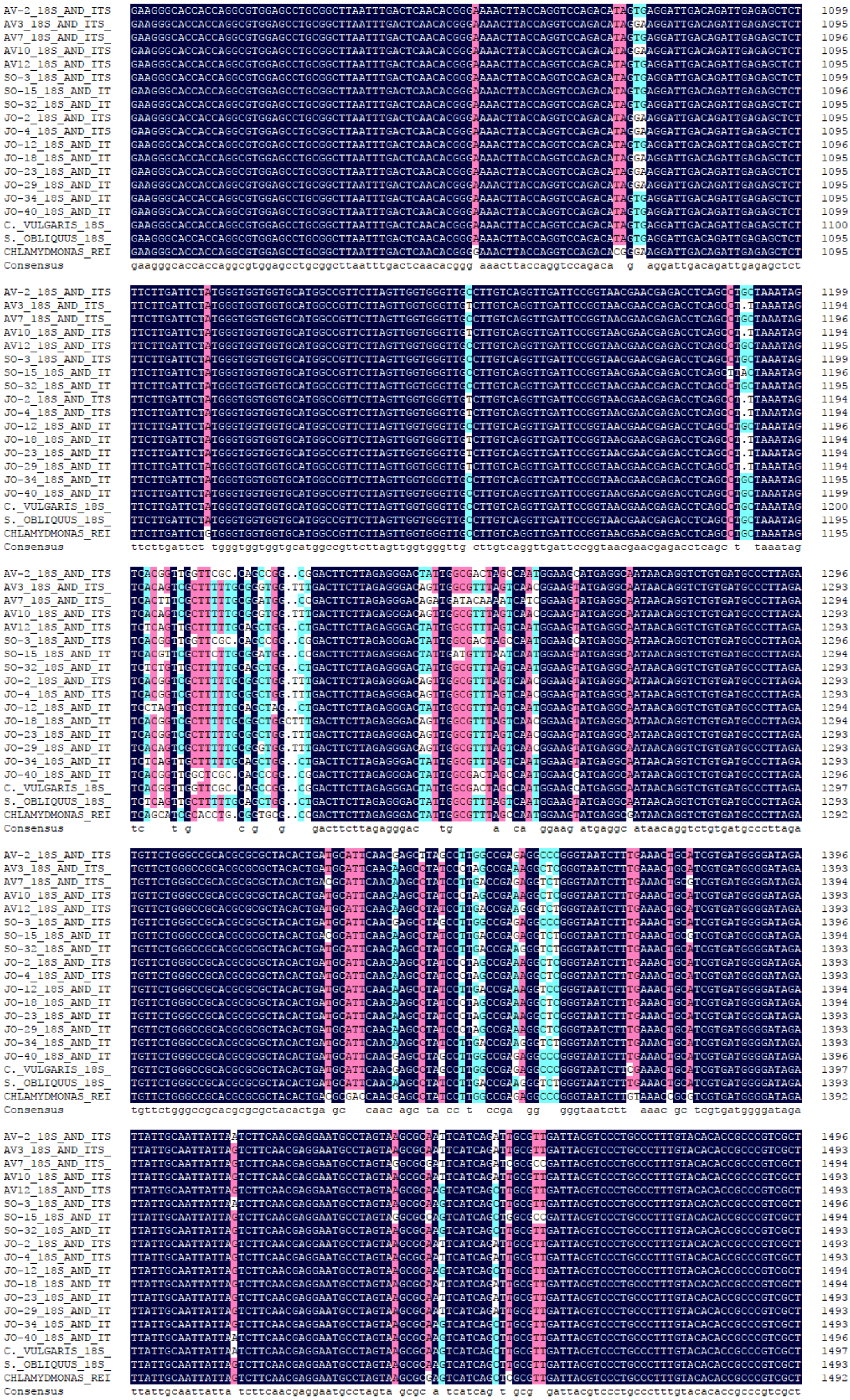
S._OBLIQUUS   TTGTGGACTTTGCTAGAGGCCAAGCAGGAAACATGCTTTGCATGTCTTAAACTTTCGACCTGAGCTCAGGCAAGGCTACCCGCTGAACTTAAGCATATCA   2432
C._VULGARIS   ....................................................................................................   1798
JO_2_FULL     ....................................................................................................   2366
JO_4_FULL     ....................................................................................................   2346
JO_12_FULL    ....................................................................................................   1550
JO_18_FULL    ....................................................................................................   1570
JO_23_FULL    ....................................................................................................   1959
JO_29_FULL    ....................................................................................................   1550
JO_34_FULL    ....................................................................................................   1571
JO_40_FULL    ....................................................................................................   1600
AV_2_FULL     ....................................................................................................   1549
AV_3_FULL     ....................................................................................................   1546
AV_7_FULL     ....................................................................................................   1547
AV_10_FULL    ....................................................................................................   1546
AV_12_FULL    ....................................................................................................   1637
SO_3_FULL     ....................................................................................................   1549
SO_15_FULL    ....................................................................................................   1547
SO_32_FULL    ....................................................................................................   1593
Consensus

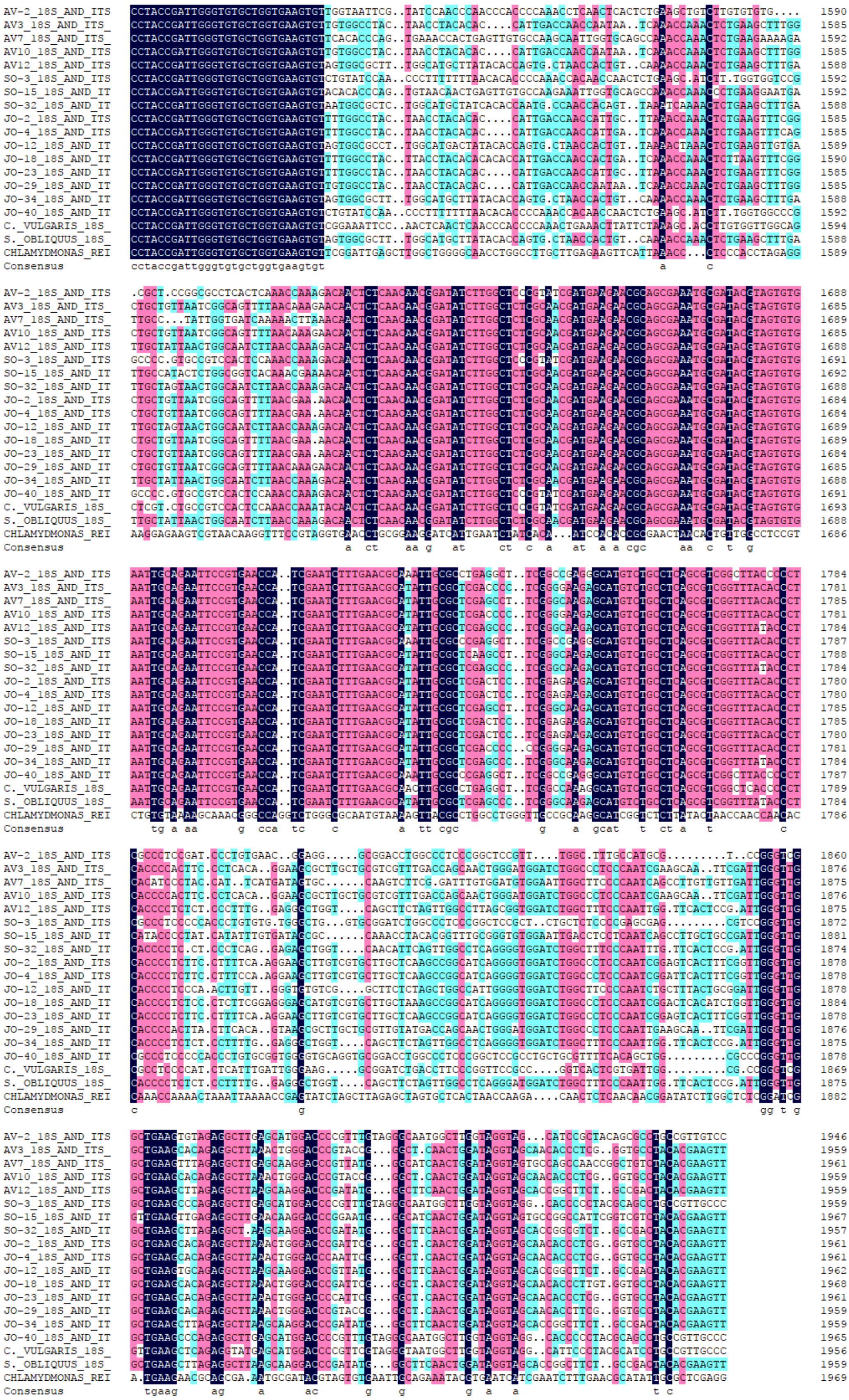
S._OBLIQUUS   ATAAGCGGAGGAAAAGAAACTAACTAGGATGCGCTTAGTAACGGCGAGCGAACCGCGCAAAGCCCAACTTGAAAATCTCCCTTGG                  2517
C._VULGARIS   .....................................................................................                  1798
JO_2_FULL     .....................................................................................                  2366
JO_4_FULL     .....................................................................................                  2346
JO_12_FULL    .....................................................................................                  1550
JO_18_FULL    .....................................................................................                  1570
JO_23_FULL    .....................................................................................                  1959
JO_29_FULL    .....................................................................................                  1550
JO_34_FULL    .....................................................................................                  1571
JO_40_FULL    .....................................................................................                  1600
AV_2_FULL     .....................................................................................                  1549
AV_3_FULL     .....................................................................................                  1546
AV_7_FULL     .....................................................................................                  1547
AV_10_FULL    .....................................................................................                  1546
AV_12_FULL    .....................................................................................                  1637
SO_3_FULL     .....................................................................................                  1549
SO_15_FULL    .....................................................................................                  1547
SO_32_FULL    .....................................................................................                  1593
Consensus
```

Appendix 1. B: Multiple alignments for the 18S rRNA and ITS1-5.8-ITS2 regions after trimming for the UK and Jordan isolates, the introns were removed from (Jo_2,4,23) 18SrRNA sequences, they were aligned with *S. obliquus*, *C. vulgaris* and *C. reinhardtii* sequences obtained from the GenBank.

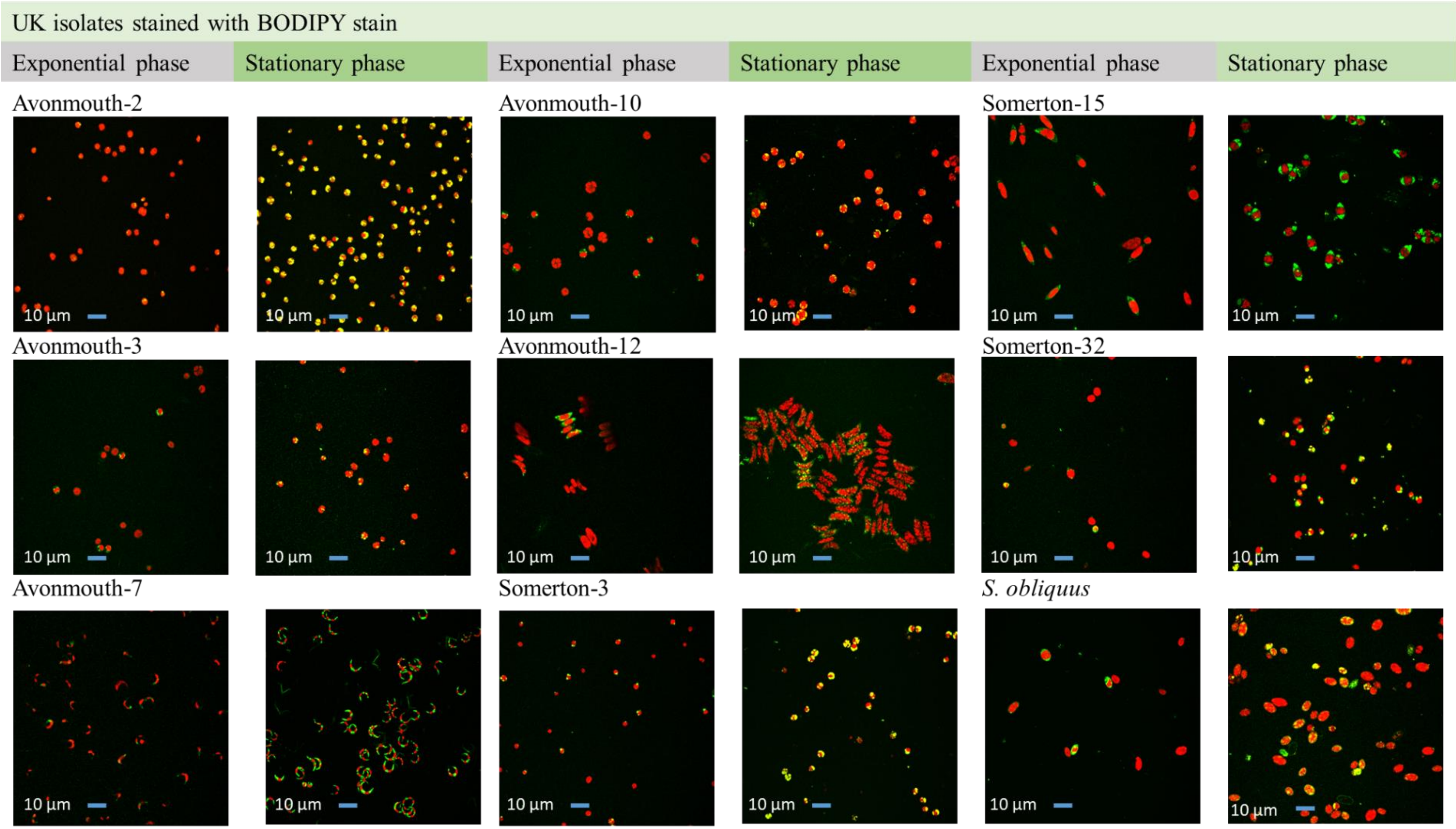


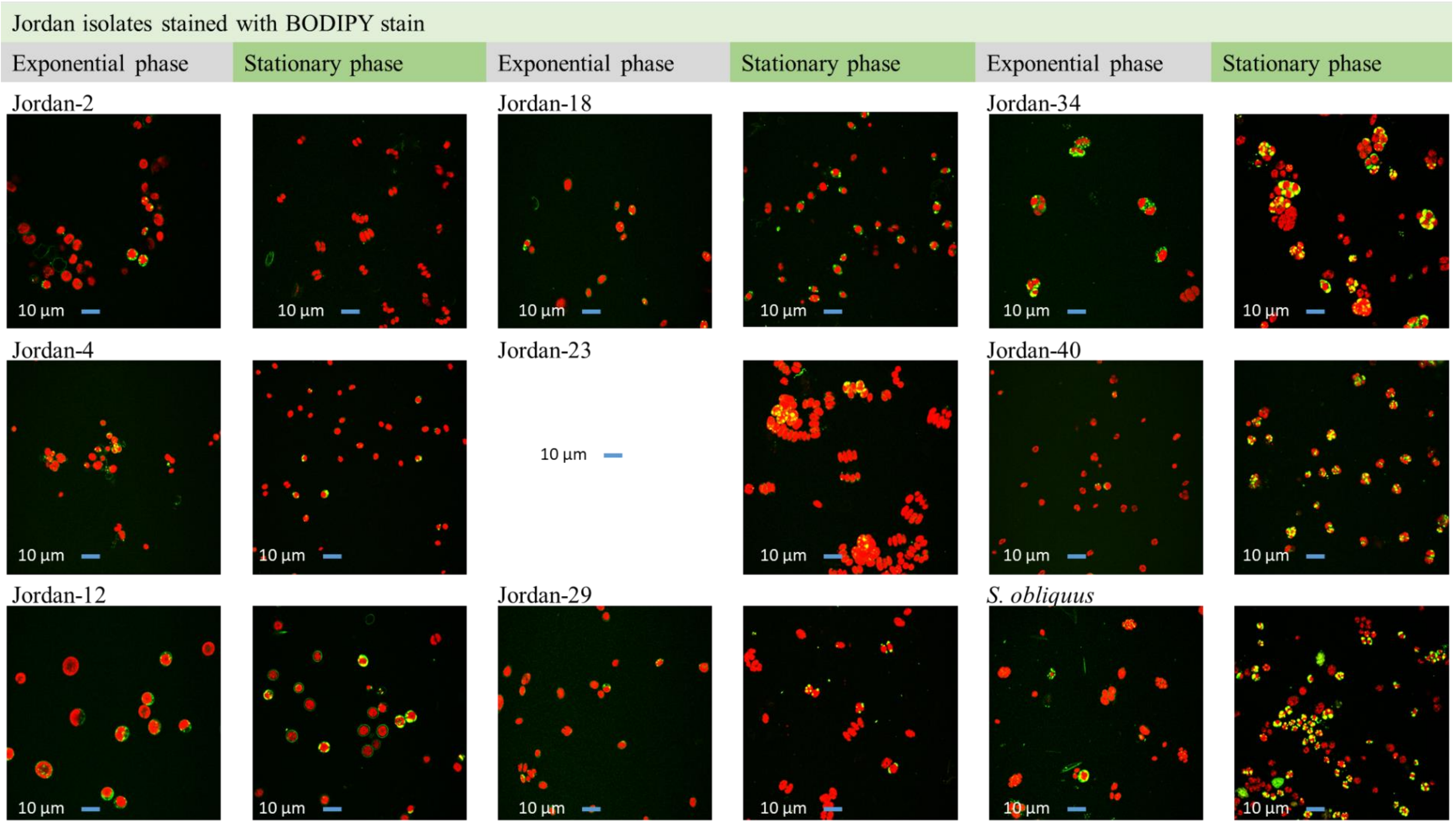

AV-2_18S_AND_ITS
AV3_18S_AND_ITS_
AV7_18S_AND_ITS_
AV10_18S_AND_ITS
AV12_18S_AND_ITS
SO-3_18S_AND_ITS
SO-15_18S_AND_IT
SO-32_18S_AND_IT
JO-2_18S_AND_ITS
JO-4_18S_AND_ITS
JO-12_18S_AND_IT
JO-18_18S_AND_IT
JO-23_18S_AND_IT
JO-29_18S_AND_IT
JO-34_18S_AND_IT
JO-40_18S_AND_IT
C._VULGARIS_18S_
S._OBLIQUUS_18S_
CHLAMYDMONAS_REI
Consensus





Appendix 2: BODIPY stain for lipid visualization in the microalgae isolates during their growth in the WW





Around 1 mL was taken from the cultures at the two growth stages stained by BODIBY stain. The samples were prepared by the addition of 1µL 1% (w/v) BODIPY $^{493/503}$ (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s- indacene) (Invitrogen) (in DMSO) and 25µL of DMSO to 100µL of cell culture, mixed well and left overnight in the fridge (dark and 4°C) and visualized by the confocal microscopy. Yellow and green florescent shows lipid in the cells, red florescent is for the chlorophyll.